1. The Rejections Under 35 U.S.C.§112, First Paragraph Should Be Withdrawn

Claims 1-19 are rejected under 35 U.S.C. §112 first paragraph. The Examiner alleges that while the specification is enabling for recombinant viral vectors such as adenovirus, lentivirus, and herpes simplex virus-1 comprising nucleic acid molecules encoding inhibitors of IL-1β and inhibitors of Fas mediated apoptosis to reduce β-cell dysfunction *in vitro*, does not reasonably provide enablement for the reduction and hence treatment for β-cell dysfunction in an individual. The Examiner asserts that the specification does not enable any person skilled in the art to which it pertains, or to which it is most nearly connected, to make or use the invention in scope with these claims.

The Examiner maintains that at the time of filing, there was no confirmed success in any human gene therapy trial, including trials involving a method of reducing β-cell dysfunction in an individual with a pancreatic disorder. According to the Examiner, at the time the invention was made, successful implementation of gene therapy was not routinely obtainable by those of skill in the art.

Applicants assert that contrary to the Examiner's contention, there have indeed recently been confirmed successes in human gene therapy trials. For example, the Examiner's attention is directed to the article of Cavazzana-Calvo et al. (2000, *Science* 288:669-672; Exhibit A) which reports on the successful use of gene therapy for the treatment of inherited severe

combined immunodeficiency (SCID). As stated on p.671, col.2, last paragraph of Cavazzana-Calvo et al., " to date, this methodology has resulted in the sustained correction (up to 10 months) of the SCID-X1 phenotype in two patients." Applicants assert that the data presented clearly demonstrates the successful use of gene therapy to provide full correction of disease phenotype and, hence, clinical benefit (see Abstract; Exhibit A).

In addition, in the same issue of *Science* reporting on the successful use of gene therapy for the treatment of SCID, is a review article that references recent publications that suggest progress in gene therapy for the treatment of hemophilia and for stimulation of new blood vessels as a means for treating cardiovascular diseases. Furthermore, early data demonstrate headway in the development of gene-based vaccines for treating several chronic infectious diseases and some types of cancers (see, Anderson, 2000, *Science* 288:627-629; Exhibit B).

The Examiner alleges that the specification fails to provide an enabling disclosure for (i) nucleic acid molecules encoding the inhibitors of IL-1β; (ii) the use of promoters used in the instant invention; (iii) methods for delivery of genes to cells in tissue culture, (iv) the number of viral particles needed to transfect cells *ex vivo*; (v) how pancreatic cells would be extracted and maintained prior to *ex vivo* transduction; and (vi) delivery of *ex vivo* transduced cells to the host.

The requirement for enablement can be found expressly stated in the first paragraph of 35 U.S.C. §112, which requires that the disclosure of an invention be "in such full,

clear, concise and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same...". The test for enablement is whether one reasonable skilled in the art could make or use the invention from the disclosure in the patent coupled with information known in the art without undue experimentation. *U.S. v Telectronics, Inc.* 857 F.2d 778, 8 USPQ2d 1217, (Fed. Cir. 1988).

The instant specification, as filed, discloses (i) specific regulators of IL-1β activity (see, page 15, line 1 through page 16, line 4 of the specification) and inhibitors of FasL triggered apoptosis (see, page 16, lines 5-17 of the specification); (ii) methods for deriving nucleic acid molecules encoding such regulators and inhibitors (see, page 18, line 3 through page 19, lin2); and (iii) recombinant expression vectors that can be utilized to express such nucleic acid molecules (see, page 19, line 8 through page 23, line 7 of the specification). Further, the specification describes methods for transfer and expression of nucleic acid molecules into pancreatic β-cells (see, page 23, line10 through page 27, line 11 of the specification). Moreover, the working examples of the specification demonstrate the successful transfer of nucleic acid molecules into pancreatic β-cells. Finally, the specification teaches (i) methods for determining effective doses (see, page 28, line 1- 6 of the specification);(ii) *in vivo* methods of administering nucleic acids (see, page 27, lines 14-20 of the specification) and *ex vivo* methods of administering pancreatic cells to a recipient host (see, page 28, line 7 through page 31, line 2 of the specification); and (iii) co-administration of specific immunosuppressive agents to the recipient host to prevent graft rejection (see, page 29, lines 14-19 of the specification).

Applicants maintain that given the specific teachings of the specification, one skilled in the art could, without undue experimentation, practice the claimed methods of the invention. All that is required is that the skilled artisan, follow the teachings of the specification.

In addition, the Examiner is reminded that a patent need not teach, and preferably omits, what is well known in the art. *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 221 USPQ 481 (Fed. Cir. 1984). Applicants assert that given the teachings of the specification, and general knowledge that is well known in the art concerning, for example, (i) inhibitors of interleukin-1 activity (see, Dinarello et al., 1998, *Intern. Rev. Immunol.* 16:457-499; Exhibit C; (ii) viral vectors for use in gene therapy (see, Robbins et al. *TIBTECH*, 1998, 16:35-40; Exhibit D); and (iii) successful transfer and expression of nucleic acids within pancreatic β-cells (see, Ju et al., 1998, *Diabetologia* 41:736-739: Exhibit E; and Csete et al., 1995, *Transplantation* 59:263-268; Exhibit F) one skilled in the art could readily prepare and utilize the claimed vectors without undue experimentation.

In view of the foregoing remarks, the rejections under 35 U.S.C. § 112, first paragraph, should be withdrawn.

2. The Claimed Invention is Not Anticipated

Claims 13-17 are rejected under 35 U.S.C. 102 (b) as being anticipated by Welling et al. (1996, Human Gene Therapy 7:1795-1802; "Welling"). The Examiner alleges that Welling discloses a recombinant adenoviral vector comprising a cDNA for human IL-1 receptor

antagonist. The Examiner maintains that Applicants disclose a recombinant adenoviral vector comprising a nucleic acid molecule encoding an inhibitor of IL-1 activity, therefore, the claimed invention is anticipated by Welling.

A claim is anticipated and fails to meet the requirement of §102 when a single prior art reference discloses each and every element of the claimed invention. *Lewmar Marine, Inc. v. Barient*, 3 USPQ2d 1766 (Fed. Cir. 1987).

Applicants have canceled claims 13-19 and added new claims 20-30. The new claims are specifically directed to the following types of adenoviral vectors: (i) vectors consisting essentially of adenoviral terminal repeats required for adenovirus replication (Claim 28); or (ii) vectors comprising a modified adenovirus E2 and E4 region (Claim 29) or a modified adenovirus E3 region (Claim 30). Since Welling only discloses the use of adenoviral vectors having deletions in the viral E1A/E1B region (see, Welling, page 1796, column 2, second paragraph) and not those vectors having the characteristics specified by new claims 28-30, the claims cannot be anticipated by Welling. Accordingly, Applicants respectfully request withdrawal of the §102 rejection.

CONCLUSION

Applicants have addressed each of the rejections set forth in the Office Action. Applicants submit that each of the rejections has been overcome or obviated by the foregoing

remarks. Applicants request that the Examiner reconsider the rejections and find claims 1-12 and new claims 20- in condition for allowance.

Respectfully submitted

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43. We thank P. Damiani, J. Kane, K. Delegge, K. Cunniff, C Malcuit, and E. Milano (Advanced Cell Technology) and the staff at Trans Ova Genetics, particularly the Genetic Advancement Center Team. We also thank A. P. Soler for his help with the electron microscopy studies and K. Chapman for helpful criticism. This work was supported in part by NIH grants AG00378, AI29524, and GM56162, a grant from the National Cancer Institute of Canada, and funds from the Terry Fox Run and the Lankenau Foundation. G.M.B. is supported by the Swiss National Science Foundation.

2 February 2000; accepted 24 March 2000

Gene Therapy of Human Severe Combined Immunodeficiency (SCID)–X1 Disease

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Severe combined immunodeficiency–X1 (SCID-X1) is an X-linked inherited disorder characterized by an early block in T and natural killer (NK) lymphocyte differentiation. This block is caused by mutations of the gene encoding the γc cytokine receptor subunit of interleukin-2, -4, -7, -9, and -15 receptors, which participates in the delivery of growth, survival, and differentiation signals to early lymphoid progenitors. After preclinical studies, a gene therapy trial for SCID-X1 was initiated, based on the use of complementary DNA containing a defective γc Moloney retrovirus–derived vector and ex vivo infection of $CD34^+$ cells. After a 10-month follow-up period, γc transgene–expressing T and NK cells were detected in two patients. T, B, and NK cell counts and function, including antigen-specific responses, were comparable to those of age-matched controls. Thus, gene therapy was able to provide full correction of disease phenotype and, hence, clinical benefit.

In considering diseases that might be ameliorated by gene therapy, a setting in which a selective advantage is conferred by transgene expression, in association with long-lived transduced cells such as T lymphocytes, may prove critical. SCID-X1 offers a reliable model for gene therapy because it is a lethal condition that is, in many cases, curable by allogeneic bone marrow transplantation (*1–4*). It is caused by γc cytokine receptor deficiency that leads to an early block in T and NK lymphocyte differentiation (*1–3*). In vitro experiments of γc gene transfer have shown that γc expression can be restored (*5–7*), as well as T and NK cell development (*8–9*), while the immunodeficiency of γc^- mice can be corrected by ex vivo γc gene transfer into hematopoietic precursor cells (*10, 11*). Long-term expression of human γc has also been achieved by retroviral infection of canine bone marrow (*12*). It has been anticipated that γc gene transfer should confer a selective advantage to transduced lymphoid progenitor cells because, upon interaction with interleukin-7 (IL-7) and IL-15, the γc cytokine receptor subunit transmits survival and proliferative signals to T and NK lymphocyte progenitors, respectively (*2, 3*). This hypothesis received further support from the observation that a spontaneously occurring γc gene reverse mutation in a T cell precursor in one patient led to a partial, but sustained, correction of the T cell deficiency, including at least 1000 distinct T cell clones (*13, 14*). Spontaneous correction of the immunodeficiency has otherwise not been observed in several hundred γc-deficient SCID patients nor in γc^- mice (*2–4*).

Two patients, aged 11 months (P1) and 8 months (P2), with SCID-X1 met the eligibility criteria for an ex vivo γc gene therapy trial. SCID-X1 diagnosis was based on blood lymphocyte phenotype determination and findings of γc gene mutations resulting either in a tail-less receptor expressed at the membrane (P1) (R289 X) or in a protein truncated from the transmembrane domain that was not expressed at cell surface (P2) (a frameshift causing deletion of exon 6) (*15*). After marrow harvesting and $CD34^+$ cell separation, 9.8×10^6 and 4.8×10^6 $CD34^+$ cells per kilogram of body weight from P1 and P2, respectively, were preactivated, then infected daily for 3 days with the MFG γc vector–containing supernatant (*16*). $CD34^+$ cells (19×10^6 and 17×10^6/kg, respectively) were infused without prior chemoablation into P1 and P2, ~20 to 40% and 36% of which expressed the γc transgene as shown by either semiquantitative PCR analysis (P1) or immunofluorescence (P2). As early as day +15 after infusion, cells carrying the γc transgene were detectable by PCR analysis (*17*) among peripheral blood mononuclear cells. The fraction of positive peripheral blood mononuclear cells increased with time (Fig. 1). T lymphocyte counts increased from day +30 in P1 (who had a low number of autologous T cells before therapy), whereas γc-expressing T cells became detectable in the blood of P2 at day +60 (Fig. 2). Subsequently, T cell counts, including $CD4^+$ and $CD8^+$ subsets, increased to 1700/μl from day +120 to +150 and reached values of ~2800/μl after 8 months (Fig. 2). Transgenic γc protein expression could not be studied on P1 cells given the presence of the endogenous tail-less protein. However, semiquantitative PCR performed at day +150 showed that a high proportion of T cells carry and express the γc transgene (Fig. 1, A and B). Similar results were observed at day +275. Southern blot analysis of provirus integration in peripheral T cells from both patients revealed a smear indicating that multiple T cell precursors had been infected by the retroviral vector (*18*).

Immunofluorescence studies showed that γc was expressed on the membrane of T cells in P2. The magnitude of expression was similar to that of control cells (Fig. 3A), as found in previous in vitro gene transfer experiments (*5, 8, 9*). These results indicate that sufficient transgene expression had been achieved and that γc membrane expression is likely to be regulated by the availability of the other cytokine receptor subunits with which γc associates (*3*). Both αβ and γδ T

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Exhibit A

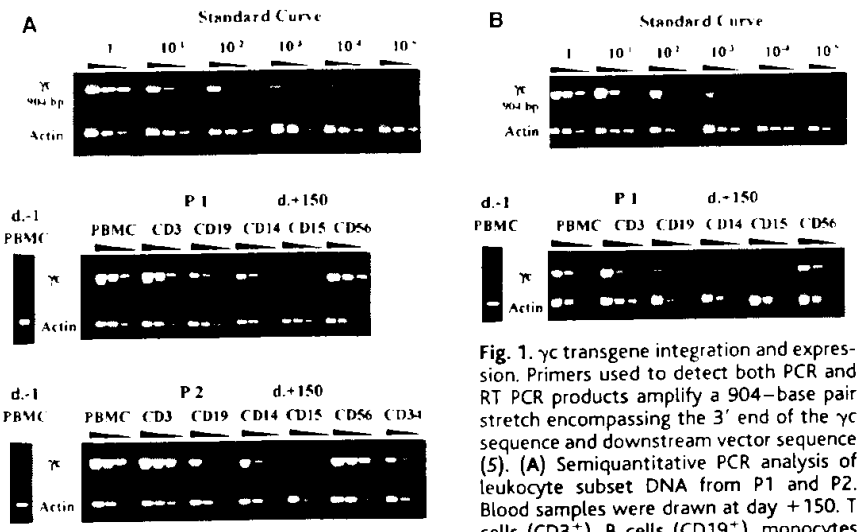


Fig. 1. γc transgene integration and expression. Primers used to detect both PCR and RT PCR products amplify a 904–base pair stretch encompassing the 3′ end of the γc sequence and downstream vector sequence (*5*). (**A**) Semiquantitative PCR analysis of leukocyte subset DNA from P1 and P2. Blood samples were drawn at day +150. T cells ($CD3^+$), B cells ($CD19^+$), monocytes ($CD14^+$), granulocytes ($CD15^+$), and NK cells ($CD56^+$) as well as $CD34^+$ from a bone marrow sample obtained at day +150 from P2 were isolated by a FACStar plus cell sorter (Becton Dickinson) after staining with appropriate mAbs (*19*). Purity was >99%. Sorted cells were analyzed for the frequency of vector-containing cells (*17*). Actin DNA was amplified in parallel. Samples from peripheral blood mononuclear cells (PBMC) obtained before treatment are shown as negative controls. A standard curve was constructed by diluting cells containing one copy of the MFG γc vector (*5*) with noninfected cells. All specimens were tested at three dilutions: 1:1, 1:20, and 1:200. (**B**) Semiquantitative RT-PCR analysis of leukocyte-subset RNA from P1. The same blood sample as in (A) was used. Actin cDNA was amplified in parallel as a control of RNA content. The standard curve was constructed as in (A) (*17*). No signal was detected in the absence of reverse transcriptase (not shown). Each specimen was diluted to 1:1, 1:500, and 1:5000.

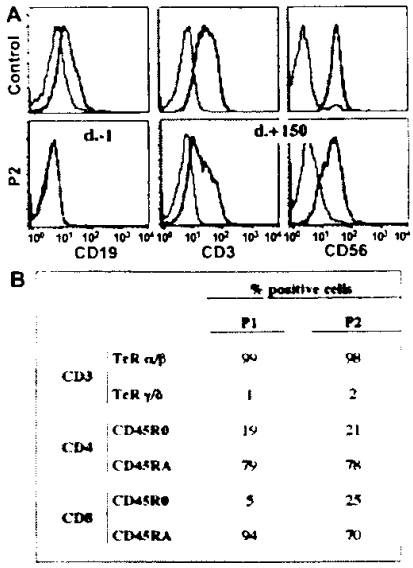


Fig. 3. γc protein expression and lymphocyte subsets. (**A**) γc protein detection at the surface of lymphocyte subsets from a control and from P2 obtained at day +150. γc expression on B cells from P2 after treatment was undetectable (not shown). The *y* axis depicts the relative cell number, and the *x* axis shows the logarithm of arbitrary immunofluorescence units. Thin lines are isotype controls; thick lines, staining by the anti-γc. Similar results were observed on blood samples obtained at days 275 (P1) and 240 (P2). (**B**) The percentage of $CD45RO^+$ and $CD45RA^+$ among CD4 and CD8 T cells from P1 and P2 obtained at day +275 and 240, respectively, as well as the percentage of T cells expressing either an αβ TCR or a γδ TCR.

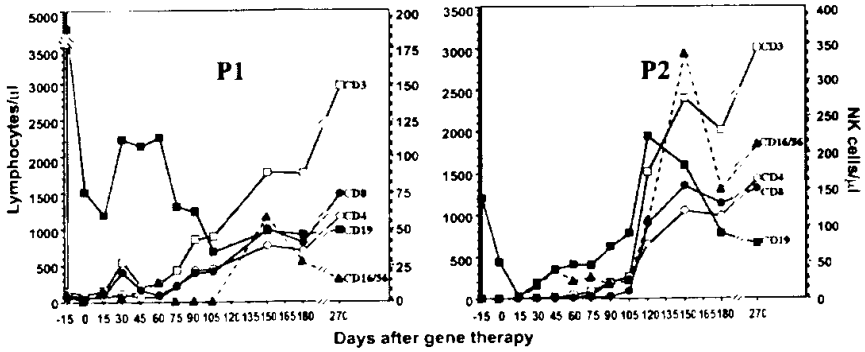
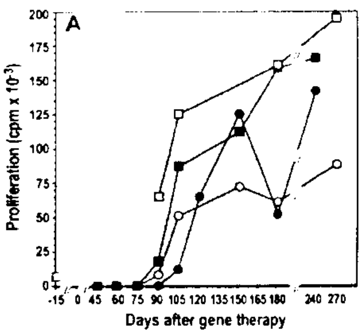


Fig. 2. Longitudinal study of lymphocyte subsets from patient 1 (P1) and patient 2 (P2). Absolute counts of T cells ($CD3^+$, $CD8^+$, and $CD4^+$), B cells ($CD19^+$), and NK cells ($CD16^+$, $CD56^+$) are shown as a function of time. Day 0 is the date of treatment. The scale for NK cells is on the right-hand side of each panel.

cell receptor (TCR)–expressing T cells were detected (Fig. 3B). Polyclonality and V_β TCR diversity were demonstrated by using antibodies specific for TCR V_β (*19*) and the immunoscope method (*18*, *20*). In both patients, naive $CD45RA^+$ T cells were detected, accounting for a majority of the T cell subset (Fig. 3B). In both patients, T cells proliferated from day +105 in the presence of phytohemagglutinin (PHA) and antibodies to CD3 (anti-CD3). The extent of proliferation was the same as that of age-matched controls (Fig. 4A). After primary vaccination, in vitro T cell proliferative responses to tetanus toxoid (P1 and P2: 18,000 and 12,000 cpm, respectively) and polioviruses (P2: 38,000 cpm) were observed within normal range (*21*). P1 T cells were also found to proliferate in the presence of protein pure derivitive (PPD) (12,000 cpm) as a likely consequence of bacillus Calmette-Guerin (BCG) persistence after immunization at 2 months of age in this immunocompromised child. Five months after cessation of intravenous immunoglobulin (Ig) therapy, antibodies to tetanus and diphtheria toxoids as well as to polioviruses were found in the serum of both patients, together with detectable concentrations of IgG and IgM (Fig. 4B). A normal level of IgA was also detected in the serum of P1. As determined by semi-quantitative PCR and reverse transcriptase–PCR analysis, it was observed that in both cases, a low fraction of B cells carry and express the γc transgene (Fig. 1). It is therefore unknown whether antibody responses are provided by untransduced or the few transduced B cells. Residual persistence (< 1%) of administered intravenous immunoglobulins (last given 5 months before measurement of antibody response) could, in part, also contribute. The γc-expressing NK cells were detected in the blood of P2 by day 30 (Figs. 1, 2, and 3A). These cells efficiently killed K562 cells in vitro (*18*). NK cells became detectable in the blood of P1 only from day +150.

As a likely consequence of development and sustained function of the immune system, clinical improvement was observed in both patients. In P2, protracted diarrhea as well as extensive graft-versus-host disease (GVHD)–like skin lesions disappeared. Both patients left protective isolation at days 90 and 95 and are now at home 11 and 10 months, respectively, after gene transfer without any treatment. Both enjoy normal growth and psychomotor development. No side effects have been noted. A similar result has since been achieved in a third patient 4 months



B				Antibodies to :		
	IgG	IgA	IgM	Tet. tox.	Dipht. Tox.	Polio virus I II III
		mg/dl		(iu/ml)		Titer x 10^{-1}
P1 (d. + 270)	323	41	202	.53	.86	320 160 160
P2 (d. + 240)	309	0	46	.93	.63	640 640 160
Control (age matched)	420-850	16-80	40-90	>.20	>.20	>80

Fig. 4. Functional characteristics of transduced lymphocyte subsets. (**A**) Longitudinal follow-up of PHA (□, ■)- and anti-CD3 (○, ●)-induced proliferation of lymphocytes from P1 (open symbols) and P2 (filled symbols) (*8*). Background [^{3}H]thymidine uptake was less than 400 cpm. Positive control values are $>50 \times 10^3$ cpm. (**B**) Serum immunoglobulin analysis was determined by nephelometry and serum antibody by enzyme-linked immunosorbent assay after immunization (see above). Diphtheria toxoid (Dipht. tox.) was also used for immunization. The last intravenous Ig injections were given at day +90 in both patients. Tet Tox., tetanus toxoid. Isohemagglutinins to blood group A have now been detected in both patients' sera.

after gene transfer (*22*). These results demonstrate that in these patients, a selective advantage was conferred to T and NK lymphocyte progenitors, enabling full-blown development of mature and functioning T and NK lymphocytes (*23*).

These overall positive results contrast with the failure of previous attempts to perform ex vivo gene therapy in adenosine deaminase (ADA)-deficient patients (*24–27*). Concomitant administration of ADA enzyme to these patients is likely to have counterbalanced the potential growth advantage of the transduced cells in this setting (*23*). Also, advances in the methodology of gene transfer into CD34$^+$ cells, i.e., the use of a fibronectin fragment (*28*) as well as of a cytokine combination enabling potent CD34 cell proliferation, contributed to the success of γc gene therapy.

Because γc gene transfer was achieved without any additional myeloablative or immunosuppressive therapy, these results pave the way for a possible extension of this therapeutic approach to other genetic diseases characterized by defective cell-subset generation, such as other forms of SCID (*29*). The kinetics of T cell development in γc gene transfer is similar to that observed in SCID patient recipients of haploidentical stem cell transplantation (*4*), suggesting that early progenitor cells have been infected by the MFG γc virus and effectively transduced. The hypothesis that transduced autologous T cells in P1 account for the development of the T cell compartment is unlikely because (i) the infected CD34$^+$ cell population was contaminated by less than 0.1% CD3$^+$ T cells; (ii) a thymic gland (27 mm by 25 mm by 25 mm at day +275) became detectable by ultrasound echography, indicative of thymopoiesis, whereas most T cells at day +275 exhibit a naïve CD45RA$^+$ phenotype; and (iii) the T cell repertoire was polyclonal and diverse. In both patients, it was shown that at day +150, a fraction of bone marrow CD34$^+$ cells harbored and expressed the γc transgene (Fig. 1, P2). It was not possible to determine whether more primitive cells, i.e., CD34$^+$CD38$^-$ cells, were transduced because of insufficient bone marrow sample. In the mouse, a common lymphoid progenitor (CLP) gives rise to the different lymphocyte populations (*30*). If a human counterpart of CLP exists, it would be the best candidate from among the earliest cells that were transduced ex vivo from these patients. Identification of integration sites in the various cell lineages could help determine the permissive differentiation stage. The question of the persistence of T and NK cell generation has yet to be addressed. If infected cells have no self-renewal capacity and have a short life-span, new generation of T and NK cells should cease. However, the fact that a thymic gland is still detectable 9 months after γc gene transfer suggests that thymopoiesis is still ongoing. Follow-up of the SCID-X1 patient in whom a spontaneous reversion mutation occurred in a T cell precursor (*13, 14*) indicates that gene transfer could be sufficient to provide a functional memory T cell pool for a number of years. This optimistic view will require careful sequential appraisal. Kohn *et al.* have previously shown that transgenes placed under the control of the long-terminal repeat (LTR) viral promoter can be silenced in quiescent T cells (*31*). Although the identification of silencing sequences in the MFG LTR makes this a strong possibility (*31*), down-regulation of γc expression has not been observed so far in these two patients, in γc-deficient mice treated by ex vivo γc gene transfer (*11*), or in cell lines maintained in culture over 1 year (*5*).

Follow-up will be required to assess the long-term effects of ex vivo γc gene transfer in CD34$^+$ cells of SCID-X1 patients. To date, this methodology has resulted in the sustained correction (up to 10 months) of the SCID-X1 phenotype in two patients, including a patient in whom the mutated protein is expressed at the cell surface. It is presumed that the effect results from a strong positive selective pressure provided to the corrected lymphoid progenitors.

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15. Patient 1 had pneumocystis carinii pneumonitis and had received BCG immunization. Patient 2 suffered from recurrent oral candidiasis, pneumocystis carinii infection, protracted diarrhea, failure to thrive, and GVHD-like skin lesions. Neither patient had an HLA (human leukocyte antigen)–identical sibling. Patients were placed in a sterile isolation ward and received nonabsorbable oral antibiotics and intravenous Igs every 3 weeks for 3 months. Parents gave informed consent for participation in the trial.
16. The defective MFG γc vector has been described previously (*5*). It was packaged in the ψcrip cell line. The MFG γc vector–containing supernatant was manufactured and provided by Genopoietic (Lyon, France) under GMP guidelines. The vector supernatant was free of replication-competent retrovirus as determined by S+L- assay and a β-galactosidase mobilization test [R. H. Bassin, N. Tuttle, P. J. Fischinger, *J. Cancer* **6**, 95 (1970); M. Printz *et al.*, *Gene Ther.* **2**, 143 (1995)]. Concentration of the virus in the supernatant was 5×10^5 infectious virus particles (*5*). Marrow CD34$^+$ cells were positively selected by an immunomagnetic procedure (CliniMACS, Miltenyi Biotec, Bergish Gladbach, Germany). CD34 cells were cultured in gas-permeable stem cell culture (PL-2417) containers (Nexell Therapeutics, Irvine, CA), at a concentration of 0.5×10^6 cells/ml in X-vivo 10 medium (Biowhittaker, Walkerville, MD) containing 4% fetal cell serum (Stem Cell Technologies, Vancouver, Canada), stem cell factor (300 ng/ml, Amgen), polyethylene glycol–megabaryocyte differentiation factor (100 ng/ml, Amgen), IL-3 (60 ng/ml, Novartis), and Flt3-L (300 ng/ml, R&D Systems, Minneapolis, MN) for 24 hours at 37°C in 5% CO_2. Containers were precoated with the CH296 human fragment of fibronectin (50 μg/ml) (TaKaRa, Shiga, Japan). Retroviral containing supernatant was added every day for 3 days. Cells were then harvested, washed twice, and infused back into the patients.
17. For semiquantitative PCR and RT-PCR analysis, DNA was isolated from the indicated cell populations. A reference standard curve was constructed by diluting cells from a SCID-X1–derived Epstein-Barr virus (EBV)–B cell line containing one copy per cell of the MFG γc provirus (*5*) in uninfected cells from the same EBV-B cell line (100, 10, 1, 0.1, 0.01, and 0.001%). DNA from each sample was also quantified by actin gel amplification. MFG γc primers sequences and

actin primers sequences are available on request. DNA was amplified in a 50 μl of PCR reaction mixture by using 30 cycles at an annealing temperature of 60°, for γc primers and 68°C for actin primers. A sample of the amplified product was separated on a 1% agarose gel and analyzed by ethidium bromide staining. RNA was prepared with the RNA easy kit (Qiagen) and was reverse-transcribed with the Superscript Preamplification System (Gibco-BRL). γc proviral and β-actin cDNA amplification were performed as described above. Quantification of expression was made by comparison with RNA isolated from the same standard curve of diluted cells.

18. M. Cavazzana-Calvo *et al.*, data not shown.
19. The following monoclonal antibodies (mAbs) were used in immunofluorescence studies: anti-γc chain: Tugh 4 (rat IgG2, PharMingen, San Diego, CA); anti-CD3: Leu 4 (IgG2a, Becton Dickinson, San Diego, CA); anti-CD4: Leu3a (IgG1, Becton Dickinson); anti-CD8: Leu 2a (IgG1, Becton Dickinson); anti-CD19: J4 119 (IgG1 Immunotech, Marseille, France); anti-CD14: Leu M3 (Becton Dickinson); anti-CD16: 3G8 (IgG1, Immunotech); anti-CD56: MY31 (IgG1, Becton Dickinson); anti-CD15 (IgM, PharMingen); anti-TCR αβ: BMA031 (IgG1, Immunotech); anti-TCR γδ: IMMU 515 (IgG1, Immunotech); anti-CD45RO: UCHL1 (IgG2a, Immunotech); anti-CD45RA: 2H4 (IgG1, Coulter Clone, Margency, France); anti-CD34: HPCA-2 (IgG1, Becton Dickinson); anti-TcR Vβ2: MPB2D5 (IgG1, Immunotech); anti-TcR Vβ3: CH92 (IgM, Immunotech); anti-TcR Vβ5.1: IMMU 157 (IgG2a, Immunotech); anti-TcR Vβ5.2: 36213 (IgG1, Immunotech); anti-TcR Vβ5.3: 3D11 (IgG1, Immunotech); anti-TcR Vβ8: 56C5.2 (IgG2a, Immunotech); anti-TcR Vβ9: FIN9 (IgG2a, Immunotech); anti-TcR Vβ13.1: IMMU 222 (IgG2, Immunotech); anti-TcR Vβ13.6: JU74.3 (IgG1, Immunotech); anti-TcR Vβ14: CAS1.13 (IgG1, Immunotech); anti-TcR Vβ17: E17.5F3.15.13 (IgG1, Immunotech); anti-TcR Vβ21.3: IG125 (IgG2, Immunotech). Fluorescence staining was done with phycoerythrin- or fluorescein isothiocyanate–conjugated mAbs. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson).
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32. We thank the medical and nursing staff of the Unité d'Immunologie et d'Hématologie pédiatriques, Hôpital des Enfants-Malades, for patient care. We also thank C. Harré and C. Jacques for technical help; D. Bresson for preparation of the manuscript; N. Wulfraat for patient referral; O. Danos, M. Fougereau, P. Mannoni, C. Eaves, and L. Coulombel for advice; A. Gennery for assistance with English translation; B. Bussière, C. CaillIot, and J. Caraux (Amgen, France) for providing SCF and MGDF; J. Bender and D. Van Epps (Nexell Therapeutics, Irvine, CA) for providing containers; and S. Yoshimura and I. Kato (Takara Shuzo, Shiga, Japan) for providing the CH-296 fibronectin fragment. Supported by grants from INSERM, Association Française des Myopathies, Agence Française du Sang, and the Programme Hospitalier de Recherche Clinique (Health Ministry).

28 December 1999; accepted 10 March 2000

REPORTS

Localization of a Short-Term Memory in *Drosophila*

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Memories are thought to be due to lasting synaptic modifications in the brain. The search for memory traces has relied predominantly on determining regions that are necessary for the process. However, a more informative approach is to define the smallest sufficient set of brain structures. The *rutabaga* adenylyl cyclase, an enzyme that is ubiquitously expressed in the *Drosophila* brain and that mediates synaptic plasticity, is needed exclusively in the Kenyon cells of the mushroom bodies for a component of olfactory short-term memory. This demonstrates that synaptic plasticity in a small brain region can be sufficient for memory formation.

The localization of memory traces has occupied neuroscientists throughout this century (*1*). Approaches have ranged from surgical ablation to mapping localized necessary gene expression in transgenic animals (*2*, *3*). Until recently, attempts to localize a memory trace have relied mainly on determining necessary brain regions (*4*). However, in a highly integrated network, other components besides the one being studied may also be necessary.

In insects, much attention has been paid to the mushroom bodies as the site for olfactory learning (*3*, *5*–*8*). In *Drosophila*, they are made up of about 2500 intrinsic neurons (Kenyon cells), receive multimodal sensory input, preferentially from the antennal lobe to the calyx, and send axon projections to the anterior brain where they bifurcate to form the α/β, α′/β′, and γ lobes (*9*). Noninvasive intervention techniques can provide mushroom body–less flies. In most respects, these flies show remarkably normal behavior but are deficient in olfactory learning (*5*). Genes important for olfactory memory have elevated expression levels in the mushroom bodies (*6*, *8*). Additionally, the mushroom bodies are necessary for context generalization in visual learning at the flight simulator and the control of spontaneous walking activity (*10*, *11*).

The *rutabaga* (*rut*) gene of *Drosophila* encodes a type I Ca^{2+}/calmodulin-dependent adenylyl cyclase (AC). Regulated synthesis of cyclic adenosine 3′,5′-monophosphate by

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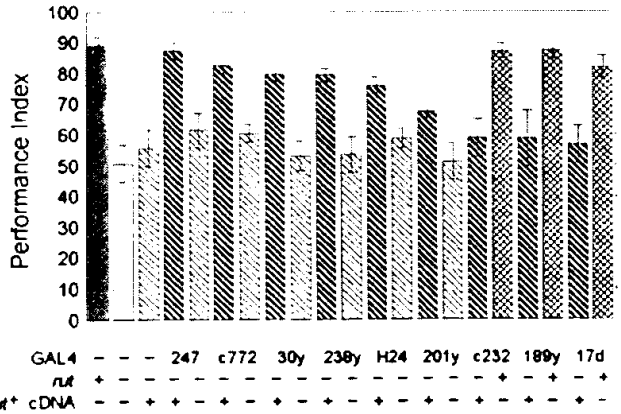

Fig. 1. The *rut* mutant defect in olfactory short-term memory can be rescued with a *rut*$^+$ cDNA in several GAL4 enhancer trap lines. Memory was measured about 2 min after classical conditioning (*17*). Performance indices (PIs) of *rut* mutant flies (white bar) and *rut* mutant flies with either a P[UAS_{GAL4}-*rut*$^+$] or GAL4 enhancer trap element (thin diagonal striped bars) were significantly different from wild-type flies (dark gray bar; P's < 0.0005). There was no significant difference between *rut* mutant flies' PIs rescued with GAL4 enhancer trap elements 247, c772, 30y, 238y, and H24 and the P[UAS_{GAL4}-*rut*$^+$] compared with wild-type flies (dark gray and thick diagonal striped bars, respectively; P's > 0.05). Mutant *rut* flies' performance was rescued with GAL4 element 201y and the P[UAS_{GAL4}-*rut*$^+$] (P < 0.05) but was also significantly lower than the performance of wild-type flies (P < 0.005). GAL4 enhancer trap lines c232, 189y, and 17d with a P[UAS_{GAL4}-*rut*$^+$] did not rescue the *rut* mutation (P's > 0.05). Wild-type flies heterozygous for GAL4 enhancer trap elements c232, 189y, and 17d were not significantly different from wild-type flies (dark gray and cross-hatched bars; P > 0.05). Bars represent mean PIs; errors are SEMs; n = 6 for all genotypes.

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GENE THERAPY:

The Best of Times, the Worst of Times

W. French Anderson*

According to recent press coverage, gene therapy has fallen on hard times. If one were to believe the news media, gene therapy is both a scientific failure and unsafe. Is this gloomy picture true? Fortunately, no. The paper by Cavazzana-Calvo *et al.* (1) on page 669 of this issue provides an example of the exciting results that are starting to be obtained in human gene therapy clinical trials. The authors have successfully treated with gene therapy (for at least up to 10 months) two infants suffering from inherited severe combined immunodeficiency (SCID).

Mutations in several different genes of immune cells can result in SCID. The first gene therapy trial almost 10 years ago treated two girls suffering from a type of SCID caused by a deficiency in the enzyme adenosine deaminase (ADA). In the new study, Cavazzana-Calvo *et al.* treat patients with an X-linked form of SCID (SCID-X1) caused by a mutation in the gene encoding the γc subunit, a component of certain cytokine receptors. After several years of preclinical studies, these investigators have carried out a clinical trial with two SCID-X1 patients, ages 11 and 8 months. They took hematopoietic stem cells (which expressed the surface marker CD34 and were capable of differentiating into all types of blood cells) from the infants' bone marrow and incubated the cells ex vivo with a retroviral vector carrying the γc cDNA. The transduced stem cells were then transfused back into the SCID-X1 patients. The authors present data from 10 months of follow-up and the results are very encouraging. Ten months after receiving transduced stem cells, the numbers of T, B, and natural killer (NK) cells of the immune system were normal, as were a number of measures of immune function (such as specific responses to antigen). Clinically, the two patients improved considerably and were able to leave protective isolation in the hospital after 3 months and have been at home ever since. Clearly, longer follow-up is necessary and more patients need to be treated, but the initial data strongly suggest that SCID-X1 can be successfully treated by retroviral-mediated gene therapy.

Why are the results of Cavazzana-Calvo *et al.* more encouraging than those from the earlier gene therapy experiments that treated ADA-deficient SCID patients (2-6)? In the first clinical protocol, the investigators inserted a normal copy of the gene encoding ADA (carried in a retroviral vector) into mature T lymphocytes (2). Later protocols attempted to transfer the same gene into bone marrow stem cells (3-5), which would differentiate into T lymphocytes capable of responding to new antigens. Cavazzana-Calvo and co-workers used a Moloney-derived retroviral vector (MFG) to deliver the therapeutic gene to the SCID-X1 infants. MFG is an improvement over the earliest retroviral vectors and may be more effective for expressing genes in T cells, but the vector itself could not be the major reason for success; MFG has been used in a number of trials without significant efficacy. Certainly, the transduction conditions of the new study are far superior to those of the early 1990s. Of most significance, perhaps, is the inclusion of Flt3 (a factor that greatly enhances stem cell growth in culture) along with other growth factors in the medium for culturing bone marrow stem cells, and the use of fibronectin-coated culture vessels. The levels of gene transduction obtained by Cavazzana-Calvo *et al.* are, consequently, much higher than those obtained in earlier studies.

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Exhibit B

Unlike the earlier gene therapy trials that treated ADA-deficient SCID patients, Cavazzana-Calvo and colleagues did not have to administer PEG-ADA--a polyethylene glycol-conjugated ADA enzyme preparation that reduces the levels of the toxic molecule deoxyadenosine in ADA-deficient patients--to the SCID-X1 infants. The concomitant administration of PEG-ADA is believed to lessen the potential growth advantage of ADA gene-corrected cells (7). Finally, SCID-X1 can result in a more profound deficiency in T cells than ADA-deficient SCID; therefore, the positive selection for gene-corrected T cells may have been more vigorous in the SCID-X1 patients.

The majority of ADA-deficient SCID patients treated with gene-corrected stem cells have not been significantly helped. But the very first ADA-deficient SCID patient, a 4-year-old girl who received only gene-corrected mature T cells, and not stem cells, has thrived (2, 8, 9) (see the figure). She received 11 infusions from September 1990 to August 1992 and has maintained a circulating level of 20 to 25% gene-corrected T cells and a normal life-style with amelioration of her disease symptoms. Her partial response to PEG-ADA treatment before gene therapy had not provided her with an adequate immune system; nonetheless, we have felt it wise to continue treating her with PEG-ADA as a safety-net.

Ashanti de Silva. Now 13, Ashanti was the first patient to be treated with gene therapy. She received infusions of T cells that had been transduced with a gene for ADA (an enzyme that she lacks), resulting in an amelioration of the symptoms of her severe combined immunodeficiency.

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Careful monitoring indicates that the vigor of her immune response has gradually diminished over the past several years, but it still remains in the normal range, albeit at the low end. But, as she only received mature gene-corrected T cells that, unlike stem cells, cannot be educated to respond to new antigens, how has she been able to generate an immune response to the new antigens that she encounters constantly? One explanation is provided by the data obtained in the first 6 months of treatment [see figure 1A of (2)]. When only a small number of gene-corrected T cells were infused (less than 1% of the total T cell population), the total number of T cells tripled from 500 to 1500 per microliter of blood. When several infusions were missed because of technical problems, the T cell number plummeted back to 500. When infusions of gene-corrected T cells were resumed, the total T cell number again rose

rapidly, this time to over 2000. One could speculate that it requires only a threshold level of "normal" (that is, gene-corrected) T cells in the lymphoid tissues to provide a microenvironment that allows the noncorrected (both immature and mature) T cells to function normally. Thus, her immature T cells may be able to differentiate in lymphoid tissue, and thereby provide her with immune protection against new antigens, as long as the "normal" mature T cells are maintained above a critical level.

The successful treatment of the first gene therapy patient suggests that the positive results of Cavazzana-Calvo *et al.* may continue over the long term. The gene-corrected stem cells of the two SCID-X1 infants should continue to experience a positive selection in the patients so that even if some cells have their γc gene silenced over time, others will expand to maintain the immune status of the patients.

In addition to the success achieved with gene therapy for the treatment of SCID, recent publications suggest progress in the treatment of hemophilia (10) and in the growth of new blood vessels to treat cardiovascular disease (11). Furthermore, early data demonstrate headway in the development of gene-based vaccines for treating several chronic infectious diseases and some types of cancer.

The field of gene therapy has been criticized for promising too much and providing too little during its first 10 years of existence. But gene therapy, like every other major new technology, takes time to develop. Antibiotics, monoclonal antibodies, organ transplants, to name just a few areas of medicine, have taken many years to mature. Major new technologies in every field, such as the manned rocket to the moon, had failures and disappointments. Early hopes are always frustrated by the many incremental steps necessary to produce "success." Gene therapy will succeed with time. And it is important that it does succeed, because no other area of medicine holds as much promise for providing cures for the many devastating diseases that now ravage humankind.

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Volume 288, Number 5466 Issue of 28 Apr 2000, pp. 627 - 629

Intern. Rev. Immunol., Vol. 16, pp. 457-499
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Interleukin-1, Interleukin-1 Receptors and Interleukin-1 Receptor Antagonist [*]

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IL-1 (IL-1α or IL-1β) is the prototypic "multifunctional" cytokine. Unlike the lymphocyte and colony stimulating growth factors, IL-1 affects nearly every cell type, and often in concert with other cytokines or small mediator molecules. Although some lymphocyte and colony stimulating growth factors may be therapeutically useful, IL-1 is a highly inflammatory cytokine and the margin between clinical benefit and unacceptable toxicity in humans is exceedingly narrow. In contrast, agents that reduce the production and/or activity of IL-1 are likely to have an impact on clinical medicine. In support of this concept, there is growing evidence that the production and activity of IL-1, particularly IL-1β, are tightly regulated events as if nature has placed specific "road blocks" to reduce the response to IL-1 during disease. In addition to controlling gene expression, synthesis and secretion, this regulation extends to surface receptors, soluble receptors and a receptor antagonist. Investigators have studied how production of the different members of the IL-1 family is controlled, the various biological activities of IL-1, the distinct and various functions of the IL-1 receptor (IL-1R) family and the complexity of intracellular signaling. Mice deficient in IL-1β, IL-1β converting enzyme (ICE) and IL-1R type I have also been studied. Humans have been injected with IL-1 (either IL-1α or IL-1β) for enhancing bone marrow recovery and for cancer treatment. The IL-1 specific receptor antagonist (IL-1Ra) has also been tested in clinical trials.

Keywords: Cytokines, IL-1β converting enzyme, Inflammation, Host defense

ABBREVIATIONS

IFN, interferon; IL, interleukin; ICE, IL-1β converting enzyme; IL-1R, IL-1 receptor; IL-1Ra, IL-1 receptor antagonist; IL-1R AcP,

[*] These studies are supported by NIH Grant AI-15614

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Exhibit C

IL-1R type I accessory protein; icIL-1Ra, intracellular IL-1Ra; LPS, lipopolysaccharide; MAP, mitogen-activated protein; NO, nitric oxide; PG, prostaglandin; ProIL-1α, IL-1α precursor; ProIL-1β, IL-1β precursor; TNF, tumor necrosis factor.

INTRODUCTION

There are three members of the IL-1 gene family: IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1Ra). IL-1α and IL-1β are agonists and IL-1Ra is a specific receptor antagonist. The naturally occurring IL-1Ra appears to be a unique situation in cytokine biology. The intron exon organization of the three IL-1 genes suggests duplication of a common gene some 350 million years ago. Before this common IL-1 gene, there may have been an ancestral gene from which fibroblast growth factor (FGF) evolved since IL-1 and FGF share significant amino acid homologies, lack a signal peptide and form an all β-pleated sheet tertiary structure. IL-1α and β are synthesized as precursors without leader sequences. The molecular weight of each precursor is 31-kD. Processing of IL-1α or IL-1β to "mature" forms of 17-kD requires specific cellular proteases. In contrast, IL-1Ra evolved with a signal peptide and is readily transported out of the cells and termed secreted IL-1Ra (sIL-1Ra).

There are two IL-1 receptors (IL-1R), the type I receptor (IL-1RI) transduces a signal whereas the type II receptor (IL-1RII) binds IL-1 but does not transduce a signal. In fact, IL-1RII acts as a sink for IL-1 and has been termed a "decoy" receptor which is somewhat unique to cytokine biology [1]. When IL-1 binds to IL-1RI, a complex is formed which then binds to the IL-1R accessory protein (IL-1R-AcP) resulting in high affinity binding [2]. It is likely that the heterodimerization of the cytosolic domains of IL-1RI and IL-1R-AcP triggers IL-1 signal transduction. The extracellular or "soluble" portions of the IL-1RI (IL-1sRI) and IL-1RII (IL-1sRII) circulate in health and disease functioning as a natural "buffers" binding IL-1α, IL-1β or IL-1Ra. In addition, several cytokines exert a negative influence on both the production and activity of IL-1.

As with other cytokines, any importance in health and disease have been revealed using gene deletion in mice. Gene expression and

synthesis of IL-1α, IL-1β or IL-1Ra has been shown in ovarian granulosa and these cells as well as in the dividing embryo. In granulosa cells from *in vitro* fertilization, there are 2000 IL-1 binding sites per cell [3]. Although IL-1 is found in placental trophoblasts and appears to play a role in embryonic development, implantation and birth of mice deficient in IL-1β, IL-1β converting enzyme or IL-1RI suggests that ovulation, fertilization, implantation and parturition either do not require IL-1 receptor signaling or that compensatory cytokines are used by these mice.

INTERLEUKIN-1α

The 31-kD IL-1α precursor (proIL-1α) is synthesized in association with cytoskeletal structures (microtubules), which is unlike most proteins translated in the endoplasmic reticulum [4]. ProIL-1α is fully active as a precursor [5] and remains intracellularly (Fig. 1). The opposite is the case with the IL-1β precursor (proIL-1β) which is not fully active and a considerable amount is secreted following cleavage by a specific, intracellular protease (see below). When cells die, proIL-1α is released and can be cleaved by extracellular proteases [6]. ProIL-1α can also be cleaved by activation of the calcium-dependent, membrane-associated cysteine proteases called calpains [7,8]. In transformed cell lines constitutively synthesizing proIL-1α, the addition of a calcium ionophore stimulates calpain which cleaves the precursor. Hence, release of the 17-kD IL-1α can take place in the absence of cell death [9].

Because of the lack of a leader peptide, proIL-1α remains in the cytosol soon after translation and there is no appreciable accumulation of IL-1 in any specific organelle. Immunohistochemical studies of IL-1α in endotoxin-stimulated human blood monocytes reveals a diffuse staining pattern but by comparison in the same cell, IL-1Ra is localized to the Golgi [10]. In experimental inflammatory bowel disease, there is a better correlation of disease severity with colonic tissue levels of IL-1α compared to those of IL-1β [11], presumably due to the cell-associated nature of IL-1α. IL-1α is not commonly found in the circulation or in body fluids except during severe disease in which case the cytokine may be released from dying cells [12] or by proteolysis after calpain-mediated cleavage [9].

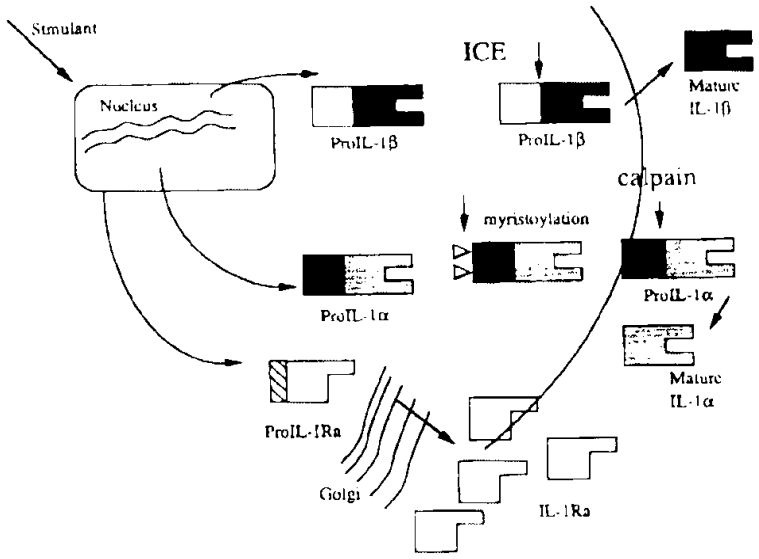

FIGURE 1 *Monocyte Producing IL-1α, IL-1β and IL-1Ra.* ProIL-1α remains in the cytosol where it is myristoylated. Myristoylated proIL-1α is translocated to the cell membrane where it can be cleaved into a mature form by the cysteine protease, calpain. Following cleavage, 17-kD IL-1α is released into the extracellular compartment. ProIL-1β remains cytosolic until cleaved by the IL-1β converting enzyme (ICE). The primary transcript is proIL-1Ra which is translated in the endoplasmic reticulum and transported to the Golgi. After cleavage of the leader sequence, IL-1Ra is readily secreted from the cell.

The concept that IL-1α can be an autocrine growth factor takes into account three distinct mechanisms: first, that proIL-1α is synthesized and remains inside the cell where it exerts a direct effect by binding to the nucleus; second, that intracellular proIL-1α complexes to an intracellular pool of IL-1RI before exerting an effect as a steroid receptor complex and thirdly, that either proIL-1α or mature IL-1α bound to surface IL-1RI is internalized with subsequent translocation to the nucleus (similar to steroid receptors). Each mechanism has supporting experimental data.

Initially, Mizel and co-workers reported that radiolabeled 17-kD recombinant IL-1α bound to the cell surface receptors, was rapidly internalized and after 2–3 h, was found associated with the nucleus [1]. Whether the nuclear binding was comprised of the

IL-1α:IL-1R complex or just the ligand. Curtis reported that internalized IL-1α was still bound to its receptor and that internalized IL-1R correlated with increased signal transduction [14]. Using truncated mutants of the cytoplasmic domain of IL-1RI, rapid internalization and nuclear localization of IL-1β was observed with several mutants not capable of transducing a biological signal [15]. It was later shown that the IL-1α,IL-1R complex but not 17-kD IL-1α bound to immobilized DNA and could be eluted under the same salt conditions as that of the estrogen receptor [16].

The cytoplasmic domain of IL-1RI is highly conserved (see below for discussion of *Toll* protein), and contains a consensus sequence (residues 517–529) similar to those that transport viral proteins [15]. If proIL-1α plays an essential role in keratinocyte cellular differentiation [17,18], it is certainly not in conjunction with the type I IL-1 receptor since mice deficient in this receptor appear to have a normal phenotype including gross examination of skin and fur (M. Labow, personal communication). The response of the IL-1RI deficient mouse to IL-1 signaling is absent (M. Labow, D. Shuster, K. McIntyre, and R. Chizzonite, unpublished observations) and it is anticipated that responses to external challenges will be similarly attenuated as those in mice treated with neutralizing antibodies to the type I receptor.

Since proIL-1α, whether recombinant [5] or natural membrane-bound [19,20], binds to the extracellular IL-1RI indistinguishably from 17-kD IL-1α, proIL-1α could also be involved in nuclear localization. Using antibodies directed specifically to proIL-1α [4] and transfection with plasmids containing the first 115 amino acids of proIL-1α (also called the IL-1α propiece), it appears that the propiece rather than the carboxyl terminal, mature segment of IL-1α localizes to the nucleus [21]. This concept is supported by the observation that a specific peptide in the propiece of IL-1α binds to DNA [22]. Phosphorylation [23] and myristoylation [24] of the IL-1α propiece may facilitate nuclear localization. Myristoylation takes place on lysine residues 82 and 83 of the IL-1α propiece which is found in the nuclear localization sequence KVLKKRR [22]. Transfecting endothelial cells with a plasmid containing this sequence revealed nuclear localization [21]. Transfecting cells with the propiece of IL-1α results in slower rate of proliferation [21] consistent with a role for IL-1α in early endothelial senescence [25]. Transfection of an intracellular IL-1 producing

plasmid increases IL-2 production in thymoma cells and this biological effect is prevented by antisense IL-1 [26], suggesting that IL-1α without its receptor is functional as an intracellular molecule.

ProIL-1α can be found on the surface of several cells, particularly on monocytes and B lymphocytes after stimulation *in vitro*. Approximately 10–15% of the IL-1α is myristoylated [24] and this form is thought to be transported to the cell surface where it is called "membrane" IL-1 [27]. Myristoylation on specific lysines facilitates passage to the cell membrane [24]. This "membrane" IL-1α is biologically active, its biological activities are neutralized by anti-IL-1α and not anti-IL-1β antibodies and appears to be anchored via a lectin interaction involving mannose residues [28]. Using high concentrations of IL-1Ra to prevent IL-1α binding to the cell surface IL-1R during fixation, the biological activity of membrane IL-1α was unaffected. In contrast, a mannose-like receptor appears to bind membrane IL-1α [19]. Although IL-1α has glycosylation sites, recombinant forms of mature IL-1 are biologically active when expressed in *E. coli* which lacks the ability to glycosylate proteins. Since membrane IL-1α is likely a glycosylated or myristoylated form of the cytokine, it accounts for no more than 5% of the total proIL-1α synthesized by the cell. There has been some dispute whether membrane IL-1α represents "leak" of intracellular IL-1 [29], but with prolonged fixation, leakage does not account for the activity of membrane IL-1 [19,30].

INTERLEUKIN-1 β

Nearly all microbes and microbial products induce production of the three IL-1 proteins but stimulants of non-microbial origin can also stimulate transcription and in many cases synthesis of the IL-1 family. Depending on the stimulant, IL-1β mRNA levels rise rapidly within 15 min but start to fall after 4 h. This decrease is thought to be due to the synthesis of a transcriptional repressor and or a decrease in mRNA half-life [31,32]. Using IL-1 itself as a stimulant of its own gene expression, IL-1β mRNA levels were sustained for over 24 h [33,34]. Raising cAMP levels in these same cells with histamine enhances IL-1α-induced IL-1β gene expression and protein synthesis [35]. In human peripheral blood mononuclear cells (PBMC), retinoic

acid induces IL-1β gene expression but the primary precursor transcripts fail to yield mature mRNA [32]. Inhibition of translation by cycloheximide results in enhanced splicing of exons, excision of introns and increased levels of mature mRNA (superinduction) by two orders of magnitude. Thus, synthesis of mature IL-1β mRNA requires an activation step to overcome an apparently intrinsic inhibition to process precursor mRNA.

Stimulants such as the complement component C5a [36], hypoxia [37], adherence to surfaces [38] or clotting of blood [39] induce the synthesis of large amounts of IL-1β mRNA in monocytic cells without significant translation into the IL-1β protein. This dissociation between transcription and translation is characteristic of IL-1β but also of TNFα [38]. It appears that the above stimuli are not sufficient to provide a signal for translation despite a vigorous signal for transcription. Without translation, most of the IL-1β mRNA is degraded and this has been observed in humans undergoing hemodialysis with complement-activating membranes [40]. Although the IL-1β mRNA assembles into large polyribosomes, there is little significant elongation of the peptide [41]. However, adding bacterial endotoxin or IL-1 itself to cells with high levels of steady state IL-1β mRNA results in augmented translation [36,38] in somewhat the same manner as the removal of cycloheximide following superinduction. One explanation is that stabilization of the AU-rich 3′ untranslated region takes place in cells stimulated with LPS. These AU-rich sequences are known to suppress normal hemoglobin synthesis. The stabilization of mRNA by microbial products may explain why low concentrations of LPS or a few bacteria or *Borrelia* organisms per cell induce the translation of large amounts of IL-1β [42].

Another explanation is that IL-1 stabilizes its own mRNA [34] by preventing deadenylation as it does for the chemokine gro-α [43]. Removal of IL-1 from cells after 2 h increases the shortening of poly (A) and IL-1 apparently is an important regulator of *gro* synthesis because it prevents deadenylation. In fact, of the several cytokines induced by IL-1, large amounts of the chemokine family are produced in response to low concentrations of IL-1. For example, 1 pM of IL-1 stimulates fibroblasts to synthesize 10 nM of IL-8 [44].

Following synthesis, proIL-1β remains primarily cytosolic until it is cleaved and transported out of the cell (Fig. 1). The IL-1β propiece

(amino acids 1–116) is also myristoylated on lysine residues [24] but unlike IL-1α, proIL-1β has no membrane form and proIL-1β is only marginally active [45]. Some IL-1β is found in lysosomes [46] or associated with microtubules [4,47] and either localization may play a role in the secretion of IL-1β. In mononuclear phagocytes, a small amount of proIL-1β is secreted from intact cells [48,49] but the pathway for this secretion remains unknown. On the other hand, release of mature IL-1β appears to be linked to processing at the aspartic acid–alanine (116–117) peptide cleavage by the IL-1β converting enzyme (ICE) [50] (see below).

Although well-controlled in the setting of laboratory cell culture, death and rupture of inflammatory cells is not an unusual occurrence *in vivo*. There are several sites in the N-terminal 16-kD part of proIL-1β which are vulnerable to cleavage by enzymes in the vicinity of alanine 117. These are trypsin [6], elastase [51], chymotrypsin [52], a mast cell chymase [53] and a variety of proteases [54,55] which are commonly found in inflammatory fluids. The extent that these proteases play in the *in vivo* conversion of proIL-1β to mature forms is uncertain but in each case, a biologically active IL-1β species is produced. In the discussion on the soluble IL-1 receptor type II (below), the affinity of proIL-1β for this constitutively produced soluble receptor is high and may prevent haphazard cleavage of the precursor by these enzymes in inflammatory fluids.

INTERLEUKIN-1 β CONVERTING ENZYME

As depicted in Fig. 1, proIL-1β requires cleavage before the mature form is secreted. The cDNA encoding ICE has been reported [56,57]. The 45-kD precursor of ICE requires two internal cleavages before becoming the enzymatically active heterodimer comprised of a 10- and 20-kD chain. The active site cysteine is located on the 20-kD chain. ICE itself contributes to autoprocessing of the ICE precursor by undergoing oligomerization with itself or homologs of ICE [58,59]. ICE is the first member of a family of intracellular cysteine proteases called caspases [60]. The term caspase was used to connote the activity of the enzymes for cleaving after an aspartic acid residue. ICE is caspase-1.

The tertiary structure of the active site has been reported [58,61]. Two molecules of the ICE heterodimer form a tetramer with two molecules of proIL-1β for cleavage [58,61]. The aspartic acid at position 116 of the proIL-1β is the recognition amino acid for ICE cleavage. ICE does not cleave the IL-1α precursor. Enzymes such as elastase [51] and granzyme A [62] cleave proIL-1β at amino acid 112 and 120 respectively yielding biologically active IL-1β. The propiece of IL-1β can be found both inside and outside the cell [63]. In addition, the propiece exhibits biological activity as a chemoattractant for fibroblasts via an IL-1R-mediated event [64].

In the presence of a tetrapeptide competitive substrate inhibitor of ICE, the generation and secretion of mature IL-1β is reduced and proIL-1β accumulates mostly inside but also outside the cell [57]. This latter finding supports the concept that proIL-1β can be released from a cell independent of processing by ICE. Similar to that of thioredoxin [65] and basic FGF [66], exocytosis has been proposed as a possible mechanism of proIL-1β release. A putative membrane "channel" where active ICE is localized has also been proposed. In this model, mature IL-1β is released through this channel [67]. When ICE activity is blocked by a reversible competitive substrate inhibitor, greater amounts of proIL-1β are found in the supernatants [57,67] and thus, the putative channel may provide a passive secretory pathway for both proIL-1β and mature IL-1β. Macrophages from ICE deficient mice do not release mature IL-1β upon stimulation *in vitro* [68,69]. Although neutrophil enzymes such as elastase and granzyme A [62] can cleave proIL-1β at sites close to alanine 117, proIL-1β accumulates in cells from ICE-deficient mice [68,69]. Interestingly, IL-1α production in macrophages from ICE deficient mice is reduced, the latter finding consistent with self-induction of IL-1 gene expression and synthesis [70]. ICE-deficient mice can be either resistant [68] or susceptible [69] to lethal endotoxemia. Recent studies suggest that mice deficient in ICE fail to develop collagen induced arthritis.

ICE AND IFN-γ -INDUCING FACTOR (IL-1δ OR IL-18?)

In 1989, an endotoxin-induced serum activity that induced interferon-γ (IFN-γ) from mouse spleen cells was described [71]. This serum

activity functioned not as a direct inducer of IFNγ but rather as a co-stimulant together with IL-2 or mitogens. An attempt to purify the activity from post-endotoxin mouse serum revealed an apparently homogeneous 50–55 kDa protein [72]. Since other cytokines can act as co stimulants for IFNγ production, the failure of neutralizing antibodies to IL-1, IL-4, IL-5, IL-6, or TNF to neutralize the serum activity suggested it was a distinct factor. In 1995, the third report was published from the same scientists demonstrating that the endotoxin-induced co-stimulant for IFNγ production was present in extracts of livers from mice preconditioned with *P. acnes* [73]. In this model, the hepatic macrophage population (Kupffer cells) expand and in these mice, a low dose of bacterial lipopolysaccharide (LPS), which in non-preconditioned mice is not lethal, becomes lethal. The factor, named IFNγ-inducing factor (IGIF), was purified to homogeneity from 1,200 grams of *P. acnes*-treated mouse livers. Its molecular weight was 18–19 kDa and a N-terminal amino acid sequence was reported [73]. Similar to the endotoxin-induced serum activity, IGIF did not induce IFNγ by itself but functioned primarily as a co-stimulant with mitogens or IL-2. Degenerate oligonucleotides derived from amino acid sequences of purified IGIF were used to clone a murine IGIF cDNA [74]. Recombinant IGIF did not induce IFNγ by itself but only in the presence of a mitogen or IL-2. However, the co-induction of IFNγ was independent of IL-12 induction of IFNγ.

Neutralizing antibodies to mouse IGIF were shown to prevent the lethality of low-dose LPS in *P. acnes* pre-conditioned mice [74]. Others had reported the importance of IFNγ as a mediator of LPS lethality in pre-conditioned mice. For example, neutralizing anti-IFNγ antibodies protected mice against Shwartzman-like shock [75] and galactosamine-treated mice deficient in the IFNγ receptor were resistant to LPS-induced death [76]. Hence, it was not unexpected that neutralizing antibodies to murine IGIF protected *P. acnes* pre-conditioned mice against lethal LPS [74]. Anti-murine IGIF treatment also protected surviving mice against severe hepatic cytotoxicity. After the murine form was cloned [74], the human cDNA sequence for IGIF was reported in 1996 [77]. Recombinant human IGIF exhibited natural IGIF activity [77]. Human recombinant IGIF was without direct IFNγ-inducing activity on human T-cells but acted as a co-stimulant for production of IFNγ and other T-helper cell-1 (Th1) cytokines [77]. IGIF induced T-cell and NK cell IFNγ production independently of IL-12 (and vice versa) [74]. To date, IGIF is thought of as primarily a co-stimulant for Th1 cytokine production (IFNγ, IL-2 and granulocyte-macrophage colony stimulating factor) [78] and also as a co-stimulant for FAS ligand-mediated cytotoxicity of murine natural killer cell clones [79]. *In vivo*, endogenous IGIF activity appears to account for IFNγ production in *P. acnes* and LPS-mediated lethality [74].

Scientists working on other IFNγ-inducing cytokines analyzed the computer generated protein folding pattern of murine IGIF and compared its pattern to those of others in the data bank. Using a validated compatibility relatedness program, the mature murine IGIF had the highest score with mature human IL-1β; furthemore, the IGIF amino acid sequence matched best with amino acids which form the all-β pleated sheet folding pattern of human IL-1β [80]. A high degree of alignment was present in the sequences that comprise the twelve β-sheets of the mature IL-1β structure. Using this alignment of conserved amino acids, there is a 19% positional identity of mature murine IGIF to mature human IL-1β and a 12% identity to human IL-1α. Using this same positional alignment, the identity of IL-1β to IL-1α is 23%. It was suggested that the name IGIF be changed to interleukin-1γ (IL-1γ) [80]. Does IGIF bind to IL-1 type I receptors? This would be an essential criterion for assigning the name IL-1γ since the type I IL-1 receptor is the signaling receptor for the biological activity of IL-1. In the absence of evidence that IGIF binds to the IL-1 receptor type I (unpublished data), IL-18 rather than IL-1γ is a more appropriate name [77]. Very little is presently known about the spectrum of its activities.

Similar to precursor IL-1β (proIL-1β), precursor IL-18 (proIL-18) does not contain a signal peptide required for the removal of the precursor amino acids with subsequent secretion. The N-terminal amino acid sequence of the secreted form of murine IL-18 [73] was consistent with that following cleavage after an aspartic acid residue, a typical cleavage site for ICE. In fact, this analysis alerted investigators that the cleavage of proIL-18 at the aspartic acid site would likely require ICE [80]. Therefore, it was not surprising that ICE cleaved proIL-18 (after the aspartic acid 19) and resulted in the mature and active protein [81].

ICE AND FAS-MEDIATED CELL DEATH

The gene *ced-3* in the nematode, *Caenorhabditis elegans*, codes for a protein homologous to human ICE [82]. During embryonic development of the worm, this gene is expressed in specialized cells and thought to be responsible for programmed cell death (apoptosis). In the worm, *ced-9* protects against apoptosis and in the human, the homologous death protecting gene is *bcl-2*. There is a remarkably conserved homology of the five amino acids required for *ced-3* and human ICE activity. Other homologs of ICE have been discovered and each has a similar aspartic acid substrate specificity. *Nedd 2* is a mouse gene also expressed in cells undergoing apoptosis during development and recently shown to be homologous to *ced-3* and ICE [83]. Overexpression of ICE or any of its homologs in transiently transfected cells is associated with increased apoptosis [83–86]. Cell death induced by ICE or its homologs can also be reduced by co-transfection with *crm-A*, a cow pox viral gene coding for an inhibitor of proteases including ICE [87]. For example, transfection with *crm-A* in neurons prevents programmed cell death due to removal of nerve growth factor [88]. *Crm-A* is, however, not specific for inhibiting ICE. One highly consistent finding is that co-transfection with *crm-A* prevents cell death associated with co-transfection of several members of the ICE family. At present, no clear proteolytic substrate cascade has been identified that accounts for the initiation of apoptosis by ICE or its homologs.

Recent studies in ICE-deficient mice have shed light on the relationship of ICE to programmed cell death. In these mice, the thymus develops normally; furthermore, stressed-induced apoptosis (corticosteroids or radiation) of thymocytes and macrophages *in vitro* is also normal in these mice [68,69]. However, apoptosis in thymocytes by an activating antibody to FAS, a TNF-related receptor, is diminished in ICE-deficient mice [69]. Blocking ICE activity with a specific substrate inhibitor also reduces FAS-induced as well as TNF-induced apoptosis [89]. Apoptosis triggered by activating FAS or TNF receptors is associated with a "death domain" on the cytoplasmic segment of these receptors. However, recent studies suggest that ICE (caspase-1) is not required for FAS-mediated cell death [90].

Several reports demonstrate that overexpressing ICE leads to programmed cell. If there is a link between ICE, IL-1 and cell death,

it is most likely due to an IL-1R-mediated event rather than an intracellular mechanism. In any cell or tissue in which IL-1β is produced under conditions of disease, agents inhibiting ICE will probably reduce IL-1β-mediated NO synthesis and any NO-mediated cell death. Under those conditions, inhibiting ICE should reduce cell death to the same extent as a neutralizing antibody to IL-1β or receptor blockade. Overall, the data support the concept that there are other substrates for caspases and that overexpressing the ICE family of proteases cleaves non-IL-1β intracellular proteins which trigger cell death. Since ICE-deficient mice have normal neuronal and immune cell development, this enzyme does not participate in cell death required for embryonic development.

There is concern that ICE inhibitors which reduce inflammation by inhibiting processing and secretion of IL-1β may inadvertently prolong the life of malignant cells. Is there a risk of aggravating autoimmune or malignant disease using ICE inhibitors? Data support the opposite view; ICE inhibition reduces inflammation without a change in cell death. One explanation may be that cleavage of molecules such as PARP require 50–100 fold more ICE compared to cleavage of proIL-1β [91]. Incubation of leukemic blasts from patients with acute myelogenous leukemia (AML) in the presence of ICE inhibitors reduced IL-1β secretion [92,93], reduced spontaneous proliferation [93] but did not increase cell survival. Antisense ICE also reduced spontaneous proliferation of AML cells without increasing survival [94]. These were anticipated results since IL-1β is a growth factor for AML cells [95]. Hence, inhibition of ICE in these models does not worsen but improves disease outcome. Nevertheless, the clinical benefit of reducing the release of mature IL-1β will require an inhibitor with a high degree of specificity for the cleavage sites of proIL-1β without affecting the cleavage of substrates of other members of the ICE family.

INTERLEUKIN-1 RECEPTOR ANTAGONIST

As shown in Fig. 1, proIL-1Ra, which possesses a leader sequence, is synthesized, processed and secreted from the cell. Upon stimulation with LPS, human blood monocytes initially express the gene for

sIL-1Ra [96]. During the first 4–6 h, sIL-1Ra protein can be visualized in the Golgi [10]. After 24 h, the primary transcript in these cells is icIL-1Ra, which lacking a leader peptide, stains diffusely in the cytosol and remains intracellular [10]. It has been proposed that icIL-1Ra consititutively produced in keratinocytes and epithelial cells may block the binding of IL-1α to nuclear DNA [96,97].

The primary amino acid homology of mature human IL-1β to IL-1Ra is 26% which is greater than that between IL-1α and IL-1β. Each member of the human IL-1 family is comprised of an all β strand molecule which forms an open barrel-like structure [98–100] closely related to structure of FGF [101]. Since each member of the IL-1 family binds to the IL-1RI, it is not surprising that IL-1α, IL-1β and IL-1Ra share structural similarities. How does IL-1Ra bind to IL-1RI with nearly the same affinity as IL-1α or IL-1β and yet not trigger a response? Crystal structural analysis of the IL-1R/IL-1Ra complex reveals that IL-1Ra contacts all three domains of the IL-1RI [102].

IL-1β has two sites of binding to IL-1RI. There is a primary binding site located at the open top of its barrel shape [103] which is similar but not identical to that of IL-1α [104]. There is a second site on the back side of the IL-1β molecule [103]. IL-1Ra also has two binding sites similar to those of IL-1β [98,105]. However, the back side site in IL-1Ra is more homologous to that of IL-1β than the primary binding site [105]. Thus, the present interpretation is that the back side site of IL-1Ra binds to IL-1RI and occupies the receptor. Lacking the second binding site, IL-1Ra does not trigger a signal (see below for discussion of IL-1 receptor dimerization). After IL-1Ra binds to IL-1RI-bearing cells, there is no phosphorylation of the epidermal growth factor receptor [106], a well-established and sensitive assessment of IL-1 signal transduction [107]. Moreover, when injected intravenously into humans at doses 1,000,000-fold greater than that of IL-1α or IL-1β [108,109], IL-1Ra has no agonist activity [110].

The formation of the heterodimer consisting of the IL-1RI and IL-1R accessory protein (IL-1R-AcP) [2] likely explains the failure of IL-1Ra to trigger a signal. From the stuctural differences described above between IL-1β and IL-1Ra, one can propose that the second binding site missing from the IL-1Ra is, in fact, the site which binds the accessory protein. The cross-linked complex of radiolabeled IL-1Ra to the type I receptor was not precipitated by a specific antibody

to the accessory protein [2]. As shown in Fig. 2, IL-1Ra binds to the type I receptor with same affinity as that of IL-1, but lacking the second binding site, the IL-1R-AcP does not dock to the IL-1Ra and the heterodimer is not formed. The binding of IL-1Ra to the type I receptor likely prevents or disrupts the complex between IL-1 and the type I receptor. This model implies that signal transduction takes place only when the heterodimer is formed. A triple mutation in IL-1Ra [111] may have partially reconstituted the second binding domain so that a degree of dimerization takes place between the cytosolic domains of IL-1RI and IL-1R-AcP, resulting in increased agonist activity of the mutated IL-1Ra [111]. IL-1β have resulted in molecules with greater than a 100-fold loss in biological activity but only a small decrease in IL-1RI binding [112,113].

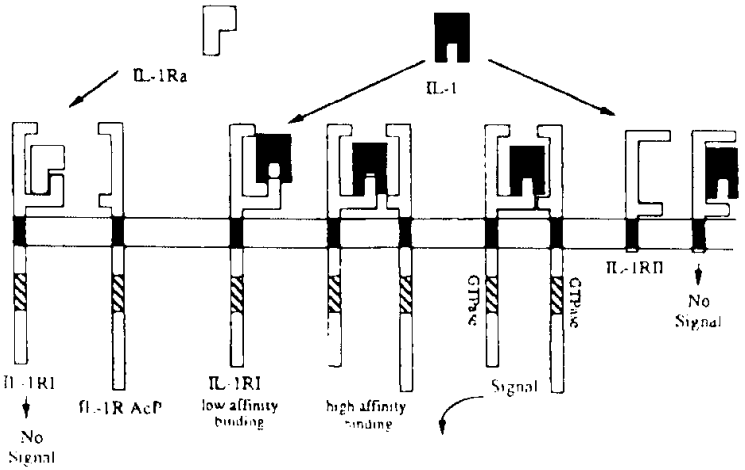

FIGURE 2 *Cellular Receptors for IL-1.* Following binding of either IL-1α or IL-1β to the IL-1RI, the IL-1R accessory protein (IL-1RAcP) forms a compex with IL-1/IL-1RI. This high affinity complex (IL-1R-AcP/IL-1/IL-1RI) results in signal transduction. Lacking a second binding site, IL-1 receptor antagonist (IL-1Ra) binds primarily to IL-1RI but does not trigger a biological response. The IL-1Ra-AcP does not form a complex with IL-1Ra/IL-1RI and no signal is transduced. Signal transduction appears to require the formation of a heterodimer of IL-1RI with IL-1R-AcP. The cytoplasmic domain of the IL-1RI contains areas with putative GTPase activity. Proteins associating with the IL-1RI cytoplasmic domains include a GTPase activating protein. The proximity of the two cytoplasmic domains likely initiates signal transduction which may include hydrolysis of GTP. The binding of IL-1 to IL-1RII does not result in signal transduction. The type II IL-1R is thought to be a "decoy" receptor.

Healthy humans are the most sensitive indicators of IL-1 agonist activity; 1 ng/kg of intravenous IL-1β produces symptoms [114]. In contrast, the intravenous infusion of 10 mg/kg of IL-1Ra in healthy humans, a 10 million-fold molar excess, is without effects [110]. What are the structural requirements of the respective molecules which account for this dramatic difference? The ability of IL-1β to optimally trigger cell signaling requires stability of the overall tertiary structure of the cytokine so that mutations in one amino acid may unfold the molecule resulting in a several hundred-fold loss in activity but without a loss in receptor binding. This suggests that biological activity requires binding of IL-1β to a relatively broad area on the receptor. The tertiary structure of IL-1Ra, which is closely related to that of IL-1β, allows for tight binding to the IL-1RI but IL-1Ra clearly lacks the second binding site which allows docking of the IL-1R-AcP to form the heterodimer. Without dimerization, no signal is transduced but occupancy of the IL-1RI by IL-1Ra results in a very effective prevention of IL-1 signal transduction. Small molecules may mimic the near perfect receptor antagonism of IL-1Ra but to date, none have been reported.

IL-1 RECEPTORS

Two primary IL-1 binding proteins (receptors) have been identified and one receptor accessory protein (IL-1R-AcP). The extracellular domains of the two receptors and the IL-1R-AcP are members of the immunoglobulin superfamily, they are each comprised of three IgG-like domains, and share a significant homology to each other [2,115,116]. Although cross-linking studies have demonstrated the existence of large molecular weight complexes with IL-1 suggesting the presence of other binding molecules [117–119], the IL-1R-AcP exhibits functionality [2]. The two IL-1 receptors are distinct gene products. In the human, the genes for IL-1RI and IL-1RII are located on the long arm of chromosome 2 [120]. Another member of the IL-1 receptor family [121] has various homologs in different species: in the rat, it is called Fit-1, an estrogen-inducible, *c-fos*-dependent transmembrane protein which shares 26–29% amino acid homology to the mouse IL-1RI and IL respectively. In the mouse, the Fit-1 protein is called

ST2 and in the human, T1. The organization of the two IL-1 receptors and the Fit-1/ST2/T1 genes indicate they are derived from a common ancestor [120]. Fit-1 exists in two forms: a membrane form (Fit-1M) with a cytosolic domain similar to that of the IL-1RI and Fit-1S, which is secreted and comprised of the extracellular domain of Fit-1M. In many ways, these two forms of the Fit-1 protein are similar to those of the membrane-bound and soluble IL-1RI. It has been shown that the IL-1sRI is derived from proteolytic cleavage of the cell bound form [120]. Although IL-1β binds weakly to Fit-1 and does not transduce a signal [122], a chimeric receptor consisting of the extracellular murine IL-1RI fused to the cytosolic Fit-1 transduces an IL-1 signal [122]. It is also unclear whether the Fit-1/ST2/T1 forms a complex with IL-1/IL-1RI/IL-1R-AcP. The cytosolic portion of Fit-1 align with GTPase-like sequences of IL-1RI [123] (see below).

IL-1RI is an 80-kD glycoprotein found prominently on endothelial cells, smooth muscle cells, epithelial cells, hepatocytes, fibroblasts, keratinocytes, epidermal dendritic cells and T lymphocytes. IL-1RI is heavily glycosylated and blocking the glycosylation sites reduces the binding of IL-1 [124]. Surface expression of this receptor is likely on most IL-1-responsive cells as biological activity of IL-1 is a better assessment of receptor expression than ligand binding to cell surfaces [125]. Failure to show specific and saturable IL-1 binding is often due to the low numbers of surface IL-1RI on primary cells. In cell lines, the number of IL-1RI can reach 5,000 per cell but primary cells usually express less that 200 receptors per cell. In some primary cells there are less than 50 per cell [126] and IL-1 signal transduction has been observed in cells expressing less than 10 type I receptors per cell [127]. The low number of IL-1RI on cells and the discrepancy between binding affinities and biological activities can be explained by the increased binding affinity of IL-1 in the complex with the IL-1R AcP [2].

As shown in Fig. 2, IL-1RI has a single transmembrane segment and a cytoplasmic domain. Using specific neutralizing antibodies, IL-1RI but not IL-1RII is the primary signal transducing receptor [127–130]. Antisense oligonucleotides directed against IL-1RI block IL-1 activities *in vitro* and *in vivo* [131]. The cytoplasmic domain of IL-1RI has no apparent intrinsic tyrosine kinase activity but when IL-1 binds to only a few receptors, the remaining unoccupied receptors appear to undergo phosphorylation [132], probably by a member of the

MAP kinase family. Interestingly, the cytosolic domain of IL-1RI has a 45% amino acid homology with the cytosolic domain of the *Drosophila Toll* gene [133]. Toll is a transmembrane protein acting like a receptor, although the ligand for the Toll protein is unknown. Gene organization and amino acid homology suggests that the IL-1RI and the cytosolic Toll are derived from a common ancestor and trigger similar signals [134,135].

As shown in Fig. 2, like other models of two chain receptors, IL-1 binds first to the IL-1RI with a low affinity. Although there is no direct evidence, a structural change may take place in IL-1 allowing docking of IL-1R-AcP to the IL-1RI/IL-1 complex. Once IL-1RI/IL-1 binds to IL-1R-AcP, a high affinity binding is observed. Antibodies to the type I receptor and to the IL-1R-AcP block IL-1 binding and activity [2]. Therefore, IL-1 may bind to the type I receptor with a low affinity causing a structural change in the ligand followed by recognition by the IL-1R-AcP. Alternatively, cells express IL-1RI IL-1R-AcP already complexed and the high affinity binding takes place on the preformed complexes.

Similar to IL-1RI and IL-1RII, a soluble form of the IL-1R-AcP exists but this form appears to result from an RNA splice donor/acceptor site resulting in a truncated protein ending before the transmembrane region. Unlike the soluble forms of the IL-1RI and IL-1RII, the soluble IL-1R-AcP is not formed by proteolytic cleavage of the full length accessory protein. It is unclear how soluble IL-1R-AcP mRNA is expressed compared to the cell-bound protein. Futhermore, since the IL-1R-AcP does not bind IL-1 itself [2], the effect of the soluble IL-1R-AcP on the binding of IL-1 remains unclear. As discussed above and shown in Fig. 2, IL-1Ra does not form a complex with the IL-1RI and this likely explains how the IL-1Ra can bind so tightly to the IL-1RI and yet not exhibit any agonist activity. One thus concludes that the IL-1RI IL-1 IL-1R-AcP complex triggers the cell and that the without the IL-1R-AcP participation, the IL-1 signal via the IL-1RI is weak or non-existent. It is unlikely that the complex IL-1RII IL-1 IL-1R-AcP exists.

The cell bound IL-1RII does not appear to form a complex with the type I receptor [128,136] nor does it transduce a signal [129,130]. In the human and mouse, IL-1RII has a short cytosolic domain consisting of 29 amino acids. The type II receptor appears to act as

"decoy" molecule, particularly for IL-1β. The receptor binds IL-1β tightly thus preventing binding to the signal transducing type I receptor [137]. It is the lack of a signal transducing cytosolic domain which makes the type II receptor a functionally negative receptor. For example, when the extracellular portion of the type II receptor is fused to the cytoplasmic domain of the type I receptor, a biological signal occurs [138]. The extracellular portion of the type II receptor is found in body fluids where it is termed IL-1 soluble receptor type II (IL-1sRII). It is assumed that a proteolytic cleavage of the extracelluar domain of the IL-1RII from the cell surface is the source of the IL-1sRII.

It is likely that as cell bound IL-1RII increases, there is a comparable increase in soluble forms [139]. Similar to soluble receptors for TNF, the extracellular domain of the type I and type II IL-1R are found as "soluble" molecules in the circulation and urine of healthy subjects and in inflammatory synovial and other pathologic body fluids [130,140–143]. In healthy humans, the circulating levels of IL-1sRII are 100–200 pM [140,141,144,145] whereas levels of IL-1sRI are 10-fold less [130,140,145]. The rank of affinities for the two soluble receptors are remarkably different for each of the three IL-1 molecules. The rank for the three IL-1 ligands binding to IL-1sRI is IL-1Ra> IL-1α>IL-1β whereas for IL-1sRII, the rank is IL-1β>IL-1α> IL-1Ra. Elevated levels of IL-1sRII are found in the circulation of patients with sepsis [144] and in the synovial fluid of patients with active rheumatoid arthritis [140], whereas the elevations of soluble type I receptor in these fluids are 10-fold lower [140]. High dose IL-2 therapy induces IL-1sRI and IL-1sRII [145].

Unlike other cytokines receptors, in cells expressing both IL-1 type I and type II receptors, there is competition to bind IL-1 first. This competition between signaling and non-signaling receptors for the same ligand appears unique to cytokine receptors, although it exits for atrial natriuretic factor receptors [146]. Since the type II receptor is more likely to bind to IL-1β than IL-1α, this can result in a diminished response to IL-1β. The soluble form of IL-1sRII circulates in healthy humans at molar concentrations which are 10-fold greater than those of IL-1β measured in septic patients and 100-fold greater than the concentration of IL-1β following intravenously administration [108]. Why do humans have a systemic response to an infusion

of IL-1β? One concludes that binding of IL-1β to the soluble form of IL-1R type II exhibits a slow "on" rate compared to the cell IL-1RI.

In addition to naturally occurring conditions which reduce a biological response to IL-1β, neutralizing antibodies to IL-1α are present in many subjects and likely reduce the activity of IL-1α. Vaccinia and cowpox virus genes encode for a protein with a high amino acid homology to the type II receptor and this protein binds IL-1β [147,148]. Despite the portfolio of soluble receptors and naturally occurring antibodies, IL-1 produced during disease does, in fact, trigger the type I receptor since in animals and humans, blocking receptors or neutralizing IL-1 ameliorates disease. These findings underscore the high functional level of only a few IL-1 type I receptors. They also imply that the post-receptor triggering events are greatly amplified. It seems reasonable to conclude that treating disease based on blocking IL-1R needs to take into account the efficiency of so few type I receptors initiating a biological event.

IL-1 ADMINISTERED TO HUMANS

IL-1α or IL-1β has been injected in patients with various solid tumors. In general, the acute toxicities of either isoform of IL-1 were greater following intravenous compared to subcutaneous injection. Subcutaneous injection was associated with significant local pain, erythema and swelling [149,150]. Most patients have been given a 15–30 min infusion with doses from 1 ng/kg to 1.0 μg/kg. Chills and fever were observed in nearly all patients, and even the 1 ng/kg dose group experienced fever [114]. The febrile response increased in magnitude with increasing doses [108,151,152]. Fever began within 30 min of starting the infusion and reached peak elevations 1.5–3 h later. When given subcutaneously, IL-1β-induced chills and fever were abated with indomethacin treatment [153]. In several studies, a transient increase in blood pressure and heart rate was observed soon after the initiation of the infusion and at the onset of the chill [152]. Following this transient increase in vascular tone, a progressive, dose-dependent fall in systolic blood pressure was observed.

In a trial of 28 patients receiving IL-1α [152] and trials of IL-1β in 19 [108] and 17 patients [151], acute toxicities of intravenously administered IL-1 were studied in detail. Nearly all patients receiving intravenous IL-1 at doses of 100 ng/kg or greater experienced significant hypotension. Systolic blood pressure fell steadily and reached a nadir of 90 mm Hg or less 3–5 h after the infusion of IL-1 [108,152,154]. At doses of 300 ng/kg, most patients required intravenous pressors. Indomethacin treatment did not reduce the hypotensive effect of IL-1, even when increased to 50 mg per day [109]. By comparison, in a trial of 16 patients given IL-1β from 4–32 ng/kg subcutaneously, there was only one episode of hypotension at the highest dose level [149].

These results suggest that the hypotension is probably due to induction of NO and elevated levels of serum nitrate have been measured in patients with IL-1-induced hypotension [152]. Despite 7 daily infusions of IL-1 and fluids for support of hypotension, there was no dramatic increase in body weight [152]. This is to be contrasted to IL-2 therapy in humans in which a significant capillary leak syndrome and weight gain is common. In addition, unlike patients on high dose IL-2 therapy, there was no increase in catheter-related infections. In patients receiving 100 ng/kg or greater of IL-1, transient dyspnea was also noted. Administration of IL-1 was associated with generalized fatigue, headache, nausea, vomiting, myalgias, arthralgias, and somnolence [108,114,152,154]. Myalgias (lower back pain) and headaches were ameliorated by indomethacin treatment. These symptoms are nearly the same as those reported by healthy volunteers receiving intravenous endotoxin, although recombinant IL-1 is essentially free of endotoxins.

Humans injected intravenously with 30–100 ng/kg of IL-1β had a sharp increase in cortisol levels 2–3 h after the injection [108,155]. Similar increases were noted in patients given IL-1α [152]. In 13 of 17 patients given IL-1β, there was a fall in serum glucose within the first hour of administration and in 11 patients, glucose fell to 70 mg/100 ml or lower [108]. In addition, there were increases in ACTH and thyroid stimulating hormone but a decrease in testosterone [152]. No changes were observed in coagulation parameters such as prothrombin time, partial thromboplastin, or fibrinogen degradation products [152]. This latter finding is to be contrasted to TNFα infusion into healthy humans which results in a distinct coagulopathy syndrome [156].

Not unexpectedly, IL-1 infusion into humans significantly increased circulating IL-6 levels in a dose-dependent fashion [152]. At a dose of 30 ng/kg, mean IL-6 levels were 500 pg/ml 4 h after IL-1 (baseline < 50 pg/ml) and 8,000 pg ml after a dose of 300 ng/kg. In another study, infusion of 30 ng kg of IL-1α induced elevated IL-6 levels within 2 h [153]. These elevations in IL-6 are associated with a rise in C-reactive protein and a decrease in albumin [152]. Serum GM CSF were less than 50 pg/ml. In two studies, one with IL-1α [158] and one with IL-1β [159], a rapid increase in circulating IL-1Ra and TNF soluble receptors (p55 and p75) were observed following a 30 min intravenous infusion. The rise in the circulating levels of both naturally occurring antagonists is greater than those measured in human volunteers injected with LPS [160,161].

Injecting humans with low-doses of either IL-1α or IL-1β confirms the impressive pyrogenic and hypotension-inducing properties of the molecules. The human studies also confirm the effects of IL-1 on stimulating the hypothalamic–pituitary–adrenal-axis and on increased cytokine production, particularly IL-6. In many ways, the signs and symptoms following IL-1 injection into humans are indistinguishable from those of low doses of endotoxin [162]. Similar to endotoxin, IL-1 induces a general enhancement of hematopoiesis, particularly in increased neutrophil, monocyte and platelet counts. In patients given marrow suppressing chemotherapy, co-treatment with IL-1 decreases the nadir and the duration of the marrow suppression. However, the benefits of IL-1 therapy in these patients are clouded by its formidable toxicity. Low doses of IL-1 may be useful in combination with other hematopoietic growth factors for reducing myelosuppression during chemotherapy or bone marrow transplantation.

IL-1 RECEPTOR ANTAGONIST AND AMELIORATION OF DISEASE

Table I lists several *in vitro* experiments in which blocking IL-1 receptors by IL-1Ra has provided a basis for animal and human studies. Because there is little species specificity, human IL-1Ra has been used in animals. Blocking IL-1 receptors with IL-1Ra has increased our understanding of IL-1 as a mediator of disease. Other [illegible] such as neutralizing anti-IL-1 antibodies, antibodies to

IL-1RI and soluble IL-1 receptors, although equally effective, are limited by their animal specificity. As listed in Table I, a reduction in the severity of various disease models has been reported. In most cases, other cytokines are produced in addition to IL-1. Therefore, the data depicted in Table I reveal that IL-1 plays an important role in the pathogenesis of inflammatory and immunologically mediated-disease. In these studies, a reduction of at least 50% is observed, but in many, the amelioration of pathological changes can be complete One consistent observation is the reduction in the number of infiltrating neutrophils associated with local inflammation and this effect of IL-1Ra may be due to preventing IL-1-induced synthesis of IL-8 and related chemokines [163].

There is, however, one important caveat: in the majority of these studies, IL-1Ra has been administered just prior to the challenging event. This is particularly the case in models of infection where injecting IL-1Ra before a lethal challenge has significantly reduced mortality but when injected shortly after the challenge, IL-1Ra had little or no effect on reducing death. On the other hand, in acute pancreatitis, a dose-dependent administration of IL-1Ra late in the disease reduced the severity of tissue damage [164,165]. In some models of chronic disease, administration of IL-1Ra after the onset of disease can still dramatically reduce severity [166]. These differences may be due to the acute or chronic nature of the model and to what extent local versus systemic IL-1 is involved.

It is not uncommon to inject high a dose of IL-1Ra (10 mg/kg) in order to observe an ameliorative effect in acute models of infection or inflammation despite the relatively low concentrations of circulating IL-1 in these animals. Why is so much IL-1Ra required? First, it should be pointed out that the plasma half-life of IL-1Ra is short (6 min) and that these models are usually "severe". For example, in the bacteremic model, the number of organisms injected intravenously ranges from 10^9 to 10^{11} per kilogram. Although systemic levels of IL-1 can be low, tissue production of IL-1 can be high [11], due to membrane IL-1α [19] and IL-1 from activated platelets [167]. In addition to these sources of IL-1, there is rapid excretion of IL-1Ra, a slow receptor "on-rate", increased IL-1RI expression, IL-1Ra binding to the soluble type I receptor and poor tissue penetration of IL-1Ra. Each can also contribute to a requirement for high doses of IL-1Ra

TABLE I Effects of IL-1Ra

Models of Infection
Improved survival in endotoxin shock in mice, rats and rabbits
Improved survival in *Klebsiella pneumoniae* infection in newborn rats
Reduction in shock and mortality in rabbits and baboons from *E. coli* or *Staphylococcus epidermidis* bacteremia
Amelioration of shock and reduction in death after cecal ligation and puncture
Attenuation of LPS-induced lung nitric oxide activity
Decreased hypoglycemia, production of CSF and early tolerance in mice after administration of endotoxin
Reduction in LPS-induced hyperalgesia
Protection against TNF-induced lethality in D-galactosamine treated mice
Reduction in nematode-induced intestinal nerve dysfunction
Decreased circulating or cellular TNF production in models of sepsis
Decreased IL-6 production after LPS
Protection from *Bacillus anthracis* toxin-induced lethality in mice
Decreased intestinal inflammation and bacterial invasion in shigellosis

Models of Local Inflammation
Decreased neutrophil accumulation in inflammatory peritonitis in mice
Reduction in immune complex-induced neutrophil infiltration, eicosanoid production and tissue necrosis in rabbit colitis
Reduction in acid-induced neutrophil infiltration and enterocolitis in rats
Decreased endotoxin-induced intestinal secretory diarrhea in mice
Reduction in ischemia and excitotoxic-induced brain damage in rats
Decrease in number of necrotic neurons in cerebral artery occlusion
Inhibition of permanganate-induced granulomas in rats
Inhibition of LPS-induced intra-articular neutrophil infiltration
Decreased IL-1-induced synovitis and loss of cartilage proteoglycan
Reduced myocardial neutrophil accumulation after coronary occlusions in dogs
Reduced inflammation and mortality in acute pancreatitis
Decreased hepatic inflammation following hemorrhagic shock

Models of Acute or Chronic Lung Injury
Decreased local LPS-induced neutrophil infiltration in rats
Inhibition of antigen-induced pulmonary eosinophil accumulation and airway hyperactivity in guinea pigs
Prevention of bleomycin or silica-induced pulmonary fibrosis
Reduction in hypoxia-induced pulmonary hypertension
Reduction in carrageenan-induced pleurisy in rats
Decreased intratracheal IL-1-induced fluid leak (systemic administration)
Decreased albumin leak after systemic LPS
Inhibition of antigen-induced eosinophil accumulation in guinea pigs

Models of Metabolic Dysfunction
Reduction in hepatocellular damage following ischemia-reperfusion
Improved survival after hemorrhagic shock in mice
Inhibition of SAA gene expression and synthesis in high dose IL-2 toxicity
Decreased muscle protein breakdown in rats with peritonitis due to cecal ligation
Reduced muscle protein breakdown in rats with chronic septic peritonitis
Inhibition of weight loss following muscle tissue injury
Decrease in bone loss in ovariectomized rats
Reversal of LPS-induced CRF gene expression in the hypothalamus
Prevention of LPS-induced ACTH release

Table I (*continued*)

Models of Autoimmune Disease
Diminution of *Streptococcus* wall-induced arthritis in rats
Reduction in collagen arthritis in mice
Suppression of anti-basement membrane glomerulonephritis
Delayed hyperglycemia in the diabetic BB rat
Reduction in streptozotocin-induced diabetes

Models of Immune-Mediated Disease
Prevention of graft versus host disease in mice
Prolongation of islet allograft survival
Reduction in autoimmune encephalomyelitis
Reduction in skin contact hypersensitivity
Decrease in coronary artery fibronectin deposition in heterotopic cardiac transplant

Models of Malignant Disease
Reduction in the number and size of metastatic melanoma
Reduction in growth of subcutaneous melanoma tumors
Reduced LPS-induced augmentation of metastatic melanoma
Reduction in tumor-mediated cachexia (intratumoral injection)

Miscellaneous
Inhibition of TNF-induced social depression in mice
Prevention of stress-induced hypothalamic monoamine release
Reduction in LPS-induced sickness behavior in rats
Suppression of crescentic glomerulonephritis in rats
Attenuation of muramyl dipeptide-induced sleep in rabbits

Impairment of Host Responses
Decreased sciatic nerve regeneration in mice
Increased mortality to *Klebsiella pneumoniae* in newborn rats (high dose)
Increased mortality to *Listeria* infection
Enhanced growth of *Mycobacterium avium* in organs
Worsening of infectious arthritis (late administration)
Increased vascular leak in mice given high dose IL-2

Studies Without an Effect of IL-1Ra
Antigen-induced arthritis in rabbits
LPS- and *Staphylococcus epidermidis* bacteremia-induced fever in rabbits
Fever after LPS injection into the brain
Leukopenia in rats after LPS
Hypertriglyceridemia after LPS in mice
LPS-induced increase in skin blood flow

It is also possible that the amount of IL-1β produced during disease has been underestimated because binding of IL-1β to the soluble (and cell-bound) type II receptor has prevented accurate measurements [140].

Since triggering so few IL-1 receptors results in a biological response, it is necessary to sustain a high level of IL-1Ra to block unoccupied receptors. When exogenous IL-1 is injected intravenously

into animals, pretreatment with a 100-fold molar excess of IL-1Ra prevents the response to IL-1. For example, injecting rabbits with 100 ng/kg of IL-1β produces fever; a pre-injection of 10 μg/kg of IL-1Ra prevents the fever. However, under natural conditions where endogenous IL-1 and other cytokines are released, an IL-1Ra plasma level of 20–30 μg/ml is needed before one observes a reduction in disease [168]. In humans, similar levels of IL-1Ra are needed to block the hematological response to LPS. *In vitro*, considerably lower concentrations of IL-1Ra are needed [169]. For example, a one to one molar ratio of IL-1Ra to IL-1 blocks 50% of the IL-1-induced response in blood monocytes [170] and a concentration of 100 ng/ml of IL-1Ra reduces the spontaneous proliferation, colony formation and cytokine production of AML or CML cells [171–173].

The molar "ratio" of endogenous IL-1Ra to IL-1β levels in body fluids from patients with infectious, inflammatory or autoimmune disease is often ten to hundred fold more IL-1Ra than IL-1β. In some selected clinical conditions, that ratio is far less. If the molar ratio of endogenous IL-1Ra to IL-1 falls, does this affect disease outcome? Some data provide important findings regarding this question. In AML cells where IL-1β is spontaneously expressed, IL-1Ra gene expression is suppressed even when stimulated with GM-CSF [173]. In 81 patients with CML, cell lysates contained more IL-1β than cells from healthy subjects whereas the levels of IL-1Ra where the same for both groups [174]. In addition, the survival of 44 patients with elevated IL-1β was lower compared to patients with low IL-1β levels. During accelerated blast crisis, IL-1Ra levels were lower compared to patients in a chronic-phase [174]. Stromal cultures established from bone marrow of patients with aplastic anemia produced less spontaneous as well as induced IL-1Ra compared to stromal cells established from normal bone marrow [175]. Recently we have measured high levels of IL-1sRII [141] in the circulation of 25 patients with hairy cell leukemia which correlate with high levels of IL-1β [176]; however, there was no increase in IL-1Ra levels in these patients.

In patients with acute Lyme arthritis, the duration of joint inflammation is shortest in those patients with the highest joint fluid levels of IL-1Ra whereas it is prolonged in those patients with low levels of IL-1Ra [177]. The reciprocal relationship was found for synovial fluid levels of IL-1β in the same patients. Similar findings were found

in the relative production of IL-1Ra and IL-1β in synovial tissue explants of patients with rheumatoid or osteoarthritis [178–180]. In normal skin, icIL-1Ra is present in higher concentrations compared to IL-1α [18] but in psoriatic lesions, the balance is in favor of IL-1α [18,181,182]. Alveolar macrophages from smokers produce less IL-1Ra than those from non-smokers [183]. Under experimental conditions, humans pretreated with corticosteroids prior to an injection of LPS produce lower circulating levels of TNF, IL-6 and IL-8 but IL-1Ra levels are unaffected by the steroids [184].

Does endogenous production of IL-1Ra affect disease outcome? There is little question that elevated production of IL-1Ra is an excellent marker of disease, and certainly a better indicator than IL-1 itself. In some clinical conditions, the elevation in IL-1Ra rather than IL-1 may indicate the presence of a pathological condition. For example, spontaneous and inducible IL-1Ra production by PBMC is higher in patients undergoing chronic hemodialysis compared to age matched patients with renal failure [185]. Detecting elevated IL-1Ra production could indicate a natural compensatory mechanism to counter the activity of IL-1, for example, in rheumatoid arthritis [186] or HIV-1-infected persons [187]. Is the amount of IL-1Ra produced in disease sufficient to dampen the response to IL-1? Using specific, neutralizing antibodies to mouse IL-1Ra, an increase in the formation of Schistosome egg granulomata was observed when endogenous IL-1Ra was neutralized [188]. In rabbits with immune complex colitis, infusion of a neutralizing antibody to rabbit IL-1Ra resulted in exacerbation and prolongation of the colitis [189]. As of this writing, the phenotype of an IL-1Ra deficient mouse is unknown but neutralizing endogenous IL-1Ra appears to worsen inflammation.

IL-1Ra ADMINISTERED TO HEALTHY HUMANS

IL-1Ra given intravenously to healthy volunteers is without side effects or changes in biochemical, hematologic or endocrinologic parameters, even when peak blood levels reach 30 μg/ml and are sustained above 10 μg/ml for several hours [110]. These studies support the concept that there is no role for IL-1 in the regulation of body temperature, blood pressure or hematopoiesis in health. It is

also consistent with the failure to observe spontaneous expression of the IL-1β gene in circulating blood cells from healthy persons using sensitive PCR methods [39]. Interestingly, PBMC taken from these volunteers after receiving IL-1Ra failed to produce IL-6 when stimulated *ex vivo* with LPS [110]. A role for IL-1R on PBMC in LPS stimulated IL-6 *in vitro* has been reported [170].

In order to evaluate the effect of IL-1 receptor blockade in clinical disease under controlled experimental conditions, healthy volunteers were challenged with intravenous endotoxin and administered an infusion of IL-1Ra at the same time. Even at 10 mg/kg IL-1Ra, there was no effect on endotoxin-induced fever, although blood levels of IL-1Ra were not significantly elevated until one hour after the bolus injection of endotoxin. Humans injected with antibodies to TNF prior to endotoxin also did not have a reduction in fever (H. Michie, personal communication). In animal studies, peripheral endotoxin induces fever by triggering IL-1 induction of IL-6 synthesis in the central nervous system [190]. Since IL-1Ra does not cross the blood brain barrier, this may account for the inability of IL-1Ra to diminish endotoxin fever [191]. However, IL-1R blockade was accomplished since there was a 50% reduction in the endotoxin-induced neutrophilia and a reduction in the circulating levels of G-CSF compared to subjects injected with endotoxin plus saline [192].

Endotoxin injection suppresses the mitogen-induced proliferative response of peripheral blood mononuclear cells *in vitro*. However, in volunteers injected with endotoxin plus IL-1Ra, there was no suppression of the response [192]. Mitogen- and antigen-induced proliferation is a well-established parameter of immunocompetence and is associated with decreased production of IL-2. Similar to experimental endotoxin injection, this suppression is observed in patients with multiple trauma, sepsis, and cardiopulmonary bypass. In experimental endotoxemia and the above clinical conditions, treatment with cyclooxygenase inhibitors restores these cell-mediated immune responses [193]. This effect of cyclooxygenase inhibitors is consistent with the well-known suppressive effects of PGE_2 on IL-2 production and T-cell proliferation. Since IL-1 is a potent inducer of COX-2, it is not surprising that blocking IL-1 receptors during endotoxemia would reduce IL-1-induced PGE_2 production during endotoxemia. Thus, these studies establish that under conditions of low-dose endotoxemia,

it is possible to block IL-1-mediated responses with IL-1Ra. Those host response parameters which were unaffected by IL-1Ra are likely due to other cytokines such as TNF or IL-6 or the combination of these cytokines with IL-1.

IL-1Ra has been in given to patients with septic shock, rheumatoid arthritis, steroid resistant graft *versus* host disease and AML and CML. The initial (Phase II) trial was a randomized, placebo-controlled, open-label study in 99 patients. Patients received either placebo, or a loading bolus of 100 mg followed by a 3 day infusion of 17, 67, or 133 mg/hr IL-1Ra [194]. A dose-dependent improvement in 28 day mortality was observed; mortality was reduced from 44% in the placebo group to 16% in the group receiving the highest dose of IL-1Ra ($p = 0.015$). In that study, there was a dose-related fall in the circulating levels of IL-6 24 h after the initiation of IL-1Ra infusion. This fall in IL-6 levels is consistent with the well-established control of circulating IL-6 levels by IL-1 [195,196] and the correlation of disease severity and outcome with IL-6 levels [197]. A large Phase III trial in 893 patients revealed a trend but without a statistically significant reduction in 28 day mortality [198]. However, a retrospective analysis of 563 patients with a predicted risk of mortality of 24% or greater [199] revealed a significant reduction in 28 day mortality (45% in the placebo group and 35% in patients receiving 2 mg/kg/hr for 72 h, $p = 0.005$) [198]. A second Phase III trial using 10 grams of IL-1Ra infused over three days was undertaken but terminated during an interim analysis because a reduction in overall 28 day mortality would not likely reach statistical significance. Patient heterogeneity is thought to contribute to a failure to bridge the gap between animal and clinical data in sepsis.

IL-1Ra was initially tested in a trial in 25 patients with rheumatoid arthritis. In the group receiving a single subcutaneous dose of 6 mg/kg, there was a fall in the mean number of tender joints ($p < 0.05$) [200]. In patients receiving 4 mg/kg per day for 7 days, there was a reduction in the number of tender joints from 24 to 10, the erthrocyte sedimentation rate fell from 48 to 31 and C-reactive protein decreased from 2.9 to 1.9 μg/ml. In this group the mean plasma concentration of IL-1Ra was 660 ± 240 ng/ml [200]. In an expanded trial, IL-1Ra was given to 175 patients [201]. Patients were enrolled into the study with active disease and taking non-steroidal anti-inflammatory drugs and or up

to 10 mg/day of prednisone. There was an initial phase of three weeks of either 20, 70 or 200 mg one, three or seven times per week. Thereafter, patients received the same dose once weekly for 4 weeks. Placebo was given to patients once weekly for the entire 7 week study period. Four measurements of efficacy were used: number of swollen joints, number of painful joints, patient and physician assessment of disease severity. A reduction of 50% or greater in these scores from baseline was considered significant in the analysis. A statistically significant reduction in the total number of parameters was observed with the optimal improvement in patients receiving 70 mg per day.

A large double-blind, placebo-controlled trial of IL-1Ra in 472 patients with rheumatoid arthritis was recently completed. There were three doses: 30, 75 and 150 mg/day for 24 weeks. There was a dose-dependent reduction in the number of swollen joints and the overall assessment of patient scores ($p = 0.048$) [202]. In addition, there was a fall in C-reactive protein and sedimentation rate. In this trial, there was a 50% reduction in new bone erosions [203].

A Phase I-II trial of escalating doses of IL-1Ra in 17 patients with steroid-resistant graft versus host disease has been completed [204]. IL-1Ra (400 to 3,400 mg/day) was given as a continuous intravenous infusion every 24 h for 7 days. Using an organ specific, acute disease scale, there was improvement in 16 of the 17 patients. Moreover, a decrease in the steady state mRNA for TNF-α in peripheral blood mononuclear cells correlated with improvement ($p = 0.001$) [204]. These studies in humans are similar to the use of IL-1Ra in animal models of graft versus host disease [205].

For clinical efficacy, IL-1Ra in patients with rheumatoid arthritis exhibits a dose-dependent response. Even the reduction of endotoxin-induced neutrophilia in healthy subjects is dose-dependent. Animal studies support these clinical observations. The requirement for such high plasma levels of IL-1Ra is not completely understood because IL-1Ra levels are already several logs higher than measurable IL-1 levels in the most severe cases of septic shock [197]. Rapid renal clearance, binding to the soluble form of the type I receptor and increased type I receptor expression may explain a need for these high levels. In addition, paracrine effects and IL-1 activity on platelets and monocytes may be binding to type I receptors on cells outside the circulation before tissue levels IL-1Ra match those in the circulation.

In the two Phase III trials of IL-1Ra in septic shock, retrospective analysis revealed decreased mortality in patient subgroups, particularly during the first seven days following entry into the trials. These results suggest that not all patients with life-threatening septic shock benefit from IL-1Ra and that factors other than IL-1 contribute to the cause of death 28 days later. Similar conclusions have been made using antibodies to TNF in clinical trials of septic shock.

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Viral vectors for gene therapy

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Gene therapy is now being applied to the treatment of a wide variety of acquired and inherited diseases. One of the rate-limiting steps for successful gene therapy is the efficiency of gene transfer. A number of different viral systems are being developed for use as vectors for *ex vivo* and *in vivo* gene transfer, including retroviruses, adenoviruses, herpes-simplex viruses and adeno-associated viruses. These viral vectors have a number of specific advantages and disadvantages that make them suited to particular gene-therapeutic applications. This review will summarize the current status of the development of viral vectors for gene therapy.

Gene therapy, the treatment or prevention of disease by gene transfer, has been a rapidly developing field of research[1–4]. Over the past ten years, gene therapy has been moving quickly from the laboratory to the clinic. Although the majority of the therapeutic trials to date have been phase 1, there is reason for optimism about the future of gene therapy. Clinical responses have been noted even in these current phase-1 studies, which are designed to test safety and feasibility rather than efficacy. As the relatively new field of gene therapy evolves, it is likely that gene-therapeutic approaches will become routine and accepted methods for treating both acquired and inherited diseases.

There are two approaches that may be utilized for gene therapy – an indirect, *ex vivo* method, in which cells are modified in culture and then transplanted, and a direct, *in vivo* gene-transfer method, involving the injection of a vector. Although the identification of the appropriate therapeutic gene(s) and of the target tissue are important for successful gene therapy, the rate-limiting step is still the ability to deliver the appropriate gene efficiently to the appropriate target tissue. There are two types of vector systems used for gene-therapeutic applications – viral and nonviral. For the most part, viral vectors are more effective than nonviral vectors for achieving high-efficiency gene transfer, but they have associated problems that still hinder their application to gene therapy, such as immunogenicity, pathology, targeting and/or the duration and level of gene expression. Nonviral vectors such as liposomes and DNA conjugates are nonpathogenic, but are less effective for gene transfer *in vivo*. The current status of the viral vectors for gene-transfer applications will be discussed below.

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Retroviral vectors

There are four types of virus currently in clinical trials – retroviruses, adenoviruses, herpes-simplex viruses and adeno-associated viruses. Of these vectors, the majority of clinical trials to date have used murine leukaemia virus (MLV)-based retroviral vectors for gene transfer[5–7]. Wild-type MLV encodes three proteins, from the genes *gag*, *pol* and *env*, which are processed into a number of polypeptides important for replication, encapsidation, infection and reverse transcription. The three proteins can be provided *in trans*, allowing the generation of vectors containing only the *cis*-acting elements that are required for these processes[8]. Cell lines that express the three viral proteins stably have been generated, and are termed packaging lines[9] (Fig. 1a). These cell lines can be used to produce recombinant, replication-defective virus by either stable or transient transfection. Currently, packaging lines that give titres of greater than 10^7 infectious virus particles per ml have been developed[10]. The use of different viral-envelope proteins, such as the G protein from vesicular-stomatitis virus, has improved titres following concentration to greater than 10^9 ml^{-1} (Ref. 10).

The advantage of retroviruses is that they can stably infect dividing cells by integrating into the host DNA without expressing any immunogenic viral proteins. In theory, the integrated retroviral vector will be maintained for the life of the host cell, continuing to express the gene of interest. Their disadvantages include the facts that the MLV-based retroviral vectors require cell division for stable infection, and that their coding capacity prevents the delivery of large genes, such as that encoding dystrophin. The recent development of vectors based on lentiviruses [such as human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) or equine infectious-anaemia virus (EIAV)], which can infect certain nondividing cells, should allow the *in vivo* use of retroviral vectors for gene-therapeutic applications. Indeed, helper-virus-free

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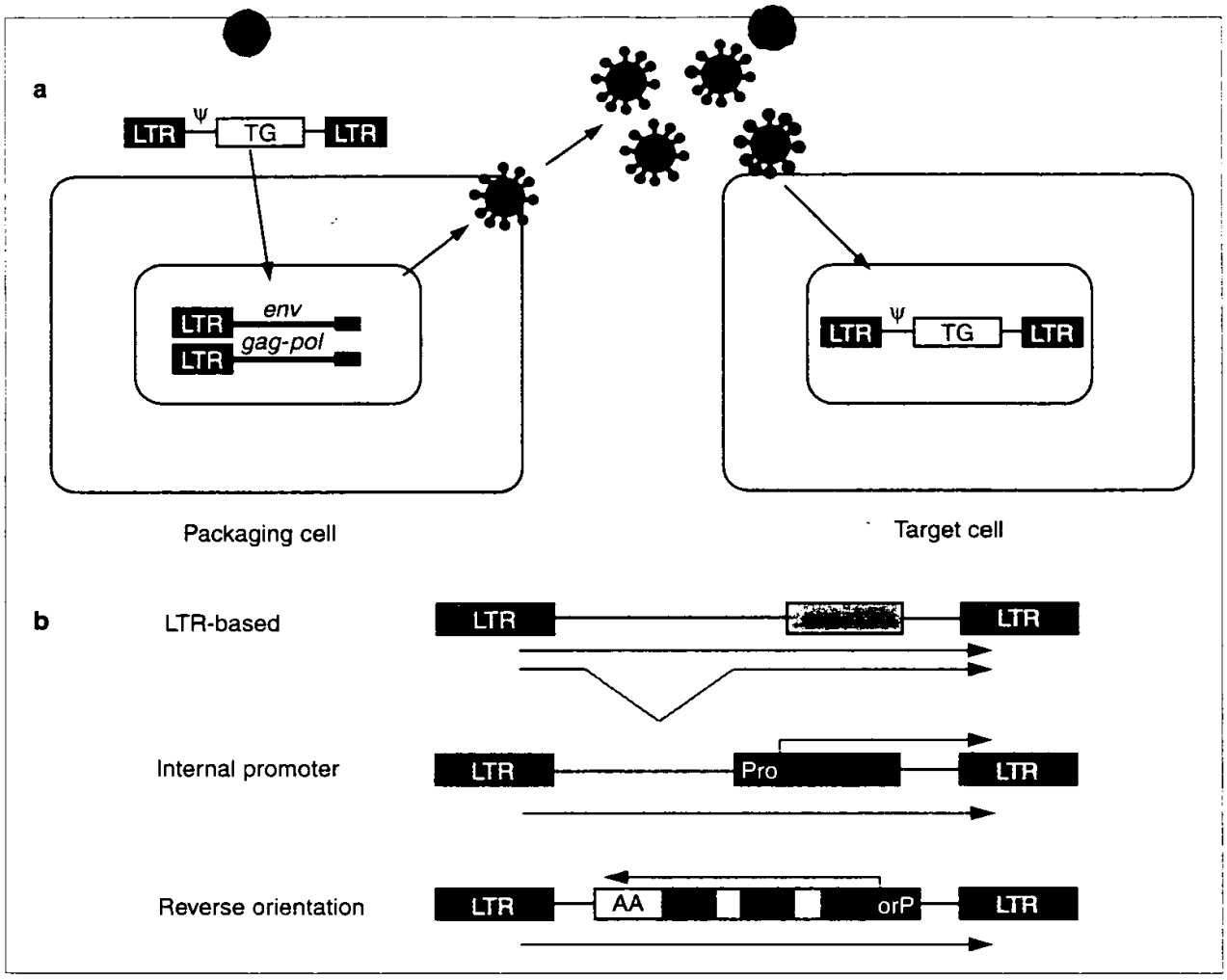

Figure 1

(**a**) Method for producing helper-free, replication-defective retroviral vectors. A packaging cell, genetically modified to express the retroviral *gag*, *pol* and *env* gene products stably, is either stably or transiently transfected with the retroviral vector containing LTRs encoding the therapeutic gene (TG). Because it is only the vector that contains a psi (Ψ) packaging sequence and two LTRs, only vector RNA is packaged into virions. Virus produced by the producer cells contains the retroviral-vector genome, which can stably infect and transduce the appropriate target cell. (**b**) Three different retroviral vector configurations can be used to express the gene of interest. For LTR-based vectors (top), initiation of transcription is regulated by the native retroviral promoter contained within the long terminal repeat (LTR) of the retrovirus (the site of transcription initiation and direction of RNA synthesis are indicated by the top arrow). In certain vectors, such as MFG, the gene of interest is inserted into the region of the virus normally encoding the *env* gene. For translation, the primary transcript is spliced (indicated by the bent arrow) using native retroviral splice-donor and -acceptor sequences. For vectors with an internal promoter (middle), transcription is driven primarily by an exogenous promoter sequence inserted immediately upstream of the coding region of the gene of interest. However, there is also a level of RNA synthesis initiated from the native promoter contained within the retroviral LTR. For reverse-orientation vectors, an exogenous promoter and the therapeutic gene are inserted into the vector in the opposite polarity to that of the direction of RNA synthesis from the viral LTR. Thus, transcription of the gene of interest is driven exclusively by the exogenous promoter. The positions of the murine leukaemia virus (MLV) long terminal repeats (LTR), promoter (Pro), reversed promoter (orP), polyadenylation sites (AA) and coding regions of the therapeutic gene (blue) are indicated.

stocks of recombinant HIV-based vectors have been generated that can infect a wide range of nondividing cells, including neurons, islets and muscle cells[12–14].

There are three types of retroviral vectors currently in use[5,8,15], which are similar, independent of whether the virus is of human, murine or avian origin (Fig. 1b).

(1) The long-terminal-repeat (LTR)-based vector, in which the therapeutic gene is expressed from the promoter in the 5′ LTR. An example of this type of vector is MFG, in which the therapeutic gene is inserted in place of the viral *env* gene[15,16]. The inserted gene is expressed from an LTR-driven, spliced message that has a 5′ untranslated sequence identical to the *env* message. Additional genes can be expressed from a polycistronic message in this vector using internal ribosome entry sites (IRESs). Indeed, up to three genes have been effectively expressed in MFG using two IRES elements[17,18].

(2) The internal-promoter vector, in which the therapeutic gene and/or a marker gene is expressed from an internal heterologous promoter. Additional genes can be expressed from the LTR, or the LTR can be mutated to prevent expression following infection of a target cell. The internal-promoter vectors containing

tissue-specific promoters can be used to facilitate high-level gene expression in specific cell types.

(3) The reverse-orientation vector, in which a genomic sequence containing a promoter, introns and polyadenylation signal can be inserted. For example, the β-globin gene has been inserted into a retroviral vector in the reverse orientation, so that the regulatory sequences in the 5′ promoter, introns and regions 3′ to the coding sequence can be maintained[19]. In this way, it is possible to achieve tightly regulated, tissue-specific gene expression.

Adenoviral vectors

Adenoviruses are linear double-stranded DNA viruses 30–35 kb in size that can cause upper-respiratory and eye infections in humans. The structure of human adenovirus type 5 and its different reading frames are indicated in Fig. 2. There are four different early genes expressed from the viruses after infection (E1, E2, E3 and E4), encoding polypeptides important for regulating viral and cellular gene expression, viral replication and the inhibition of cellular apoptosis. Late in infection, the major late promoter is activated, resulting in the expression of polypeptides required for encapsidation of the virus. Adenoviruses can be converted for use as vectors for gene transfer by deleting the E1 gene, which is important for the induction of the E2, E3 and E4 promoters[20]. The E1⁻, replication-defective virus can be propagated in a cell line that provides the E1 polypeptides *in trans*, such as the human embryonic kidney cell line 293. A therapeutic gene (or genes) can be inserted by recombination in place of the E1 gene; expression is driven from either the E1 promoter or a heterologous promoter (Fig. 2b).

The advantages of adenoviral vectors are that they can infect a wide variety of cell types, including non-dividing cells, and can be grown to high titres[21]. However, the viral genome remains episomal, allowing for only transient gene expression. In addition, the current first-generation E1⁻ viruses still express a low level of viral proteins after infection, resulting in a low level of viral replication. The viral gene expression can induce a CD4- and CD8-dependent immune response that reduces the duration of the gene expression *in vivo*[22,23]. In order to develop vectors that may be less immunogenic, more-defective viruses are being constructed. In particular, the deletion of some or all of the E4 open reading frames (ORFs) results in more-attenuated viruses[21,24–28]. However, certain second-generation vectors appear not to give longer-term gene expression, even though the DNA seems to be maintained. Thus, it appears that the function of one or more of the E4 ORFs may be to enhance gene expression from at least certain viral promoters carried by the virus. An alternative approach to making a more defective virus has been to 'gut' the virus completely, maintaining only the terminal repeats required for viral replication[29–31] (Fig. 2c). The 'gutted', or 'gutless', viruses can be grown to high titres with a first-generation helper virus in the 293 cell line, but it has been difficult to separate the 'gutted' vector from the helper virus.

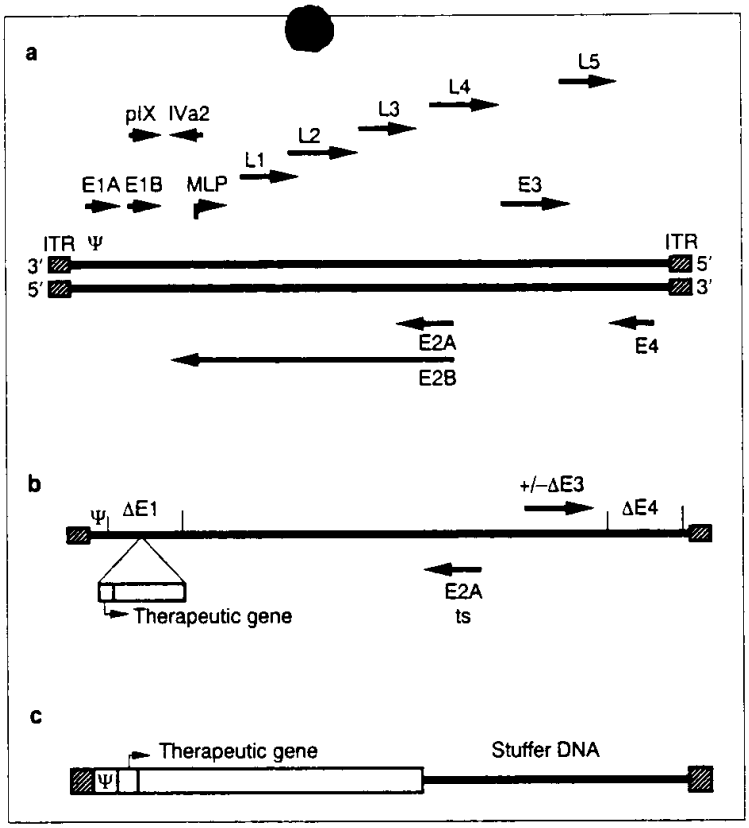

Figure 2

Structure of adenovirus type 5 (Ad5) genome. (**a**) The different transcription units for the wild-type virus are indicated by arrows. Alternative splicing leads to the RNAs for early (E), intermediate (pIX and IVa2) and late (L) genes being generated from the primary transcripts. Proteins from the early genes are required for functions such as host-cell transformation and viral-DNA replication. The late genes [transcribed from the major late promoter (MLP)] primarily encode structural proteins of the virion. Inverted-terminal-repeat (ITR) sequences function as replication origins, and the Ψ sequence is required for packaging of the viral genome. (**b**) Mutations can be introduced into the Ad5 genome to make a replication-defective virus for gene transfer. When the E1 protein is supplied *in trans* by a complementing cell line, the E1 coding region can be deleted and the therapeutic gene inserted under the regulation of a heterologous promoter. The E4 coding region has also been deleted in second-generation vectors. The E3 region, involved in blocking the immune response to the virally infected cell, can be either deleted or retained. In addition, a temperature-sensitive (ts) E2A allele has been used to make a more-defective virus. (**c**) An alternative approach to making a defective virus is to completely 'gut' it, that is, to remove all viral coding regions from the virus, leaving only the ITRs, the Ψ sequence and the therapeutic gene.

Replication-competent adenoviruses can also be used for gene therapy. For example, the E1A gene can be inserted into a first-generation virus under the regulation of a tumour-specific promoter[32]. In theory, following injection of the virus into a tumour, it could replicate specifically in the tumour but not in the surrounding normal cells. This type of vector could be used either to kill tumour cells directly by lysis or to deliver a 'suicide gene' such as the herpes-simplex-virus thymidine-kinase gene (HSV *tk*), which can kill infected and bystander cells following treatment with ganciclovir. Alternatively, an adenovirus defective only for E1B has been used specifically for antitumour

treatment in phase-[redacted]nical trials[33,34]. The polypeptides encoded by E1B ar[redacted]le to block p53-mediated apoptosis, preventing the cell from killing itself in response to viral infection. Thus in normal, nontumour cells, in the absence of E1B, the virus is unable to block apoptosis and is thus unable to produce infectious virus and spread. In tumour cells deficient in p53, the E1B-defective virus can grow and spread to adjacent p53-defective tumour cells, but not to normal cells. Again, this type of vector could also be used to deliver a therapeutic gene such as HSV *tk*.

Herpes-simplex virus

Herpes-simplex viruses I and II are large linear DNA viruses of approximately 150 kb encoding 70–80 genes (Fig. 3). The wild-type viruses are able both to infect cells lytically and to establish latency in specific cell types, such as neurons. Like adenoviruses, HSV can infect a wide variety of cell types, including muscle, tumours, lung, liver and pancreatic islets. In order to use HSV[redacted] vector, it has to be rendered replication defective. Following infection of a cell with HSV, the expression of a small number of immediate early (IE) genes is induced by a viral transactivating protein, VP16, which is carried into the cell as part of the viral tegument. The IE genes, which include ICP0, 4, 6, 22 and 27, are themselves regulators of gene expression that are important for the induction of the early and late genes required for viral replication and encapsidation[35–38]. Mutation of ICP4 results in a virus unable to replicate except in a complementing cell line, but which still expresses the other IE-gene products; these other IE proteins are toxic to many cell types. Recently, vectors defective for ICP4, 22 and 27 have been generated that have reduced levels of toxicity and prolonged gene expression in culture and *in vivo*[35–40]. The development of vectors defective for all IE genes should, in conjunction with the appropriate complementing cell lines, allow the widespread clinical application of HSV vectors.

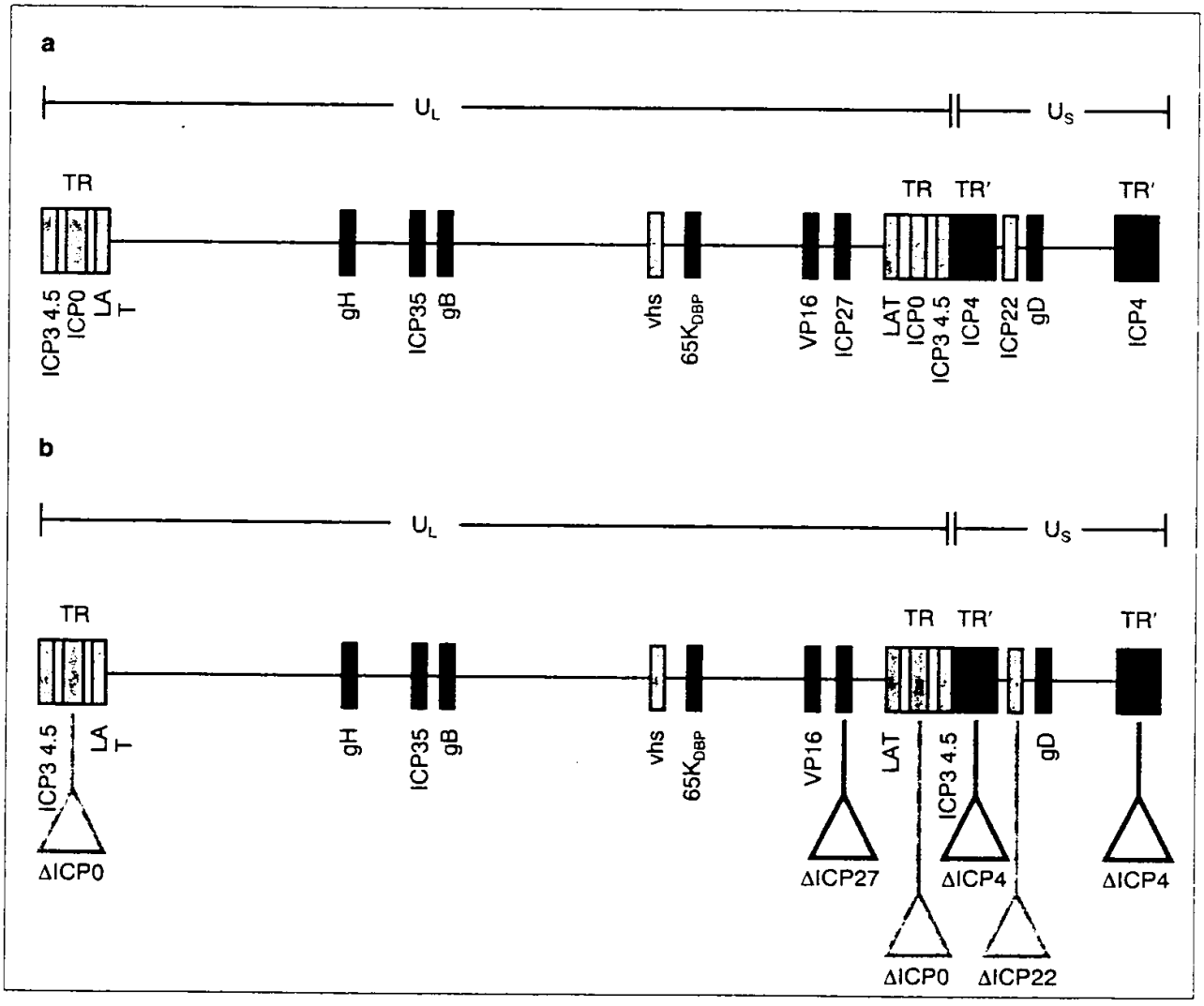

Figure 3

Structure of herpes-simplex virus. (**a**) The positions of several essential and nonessential genes are indicated, as are the unique-long (U_L) and -short (U_S) regions of the virus. (**b**) The virus can be rendered defective by deleting several of the nonessential, immediate-early genes such as ICP0, ICP4, ICP22, and ICP27. These proteins are supplied *in trans* by complementing cell lines and/or transient transfection of expression plasmids. The large size of the genome (152 kb) may allow the insertion of multiple therapeutic genes.

An alternative approach to producing infectious HSV vectors is the use of amplicons. In this approach, a plasmid containing an HSV origin of replication and packaging sequence is cotransfected with cosmids containing the HSV genome but with a defective packaging sequence[41–43]. The resulting virus contains only plasmid sequences, thereby eliminating any toxicity associated with low-level HSV-protein expression. Although this approach can generate a helper-free stock of virus, the titres are still very low.

The advantage of HSV vectors is that they have a large capacity for inserting heterologous DNA, allowing up to 50 kb to be included successfully, possibly consisting of multiple therapeutic genes. For example, four different antitumour genes have been inserted into a single HSV vector for use in cancer therapy (J. Glorioso, pers. commun.). Alternatively, HSV vectors can be used to obtain highly regulated gene expression. An RU486-hormone-regulated chimeric transcription factor has been inserted into HSV along with a promoter containing binding sites for the regulated transcription factor; specific, regulated gene expression has been observed *in vivo*.

Adeno-associated virus

Adeno-associated virus (AAV) is a member of the parvovirus family, small single-stranded DNA viruses that require a helper virus, such as adenovirus or herpes-simplex virus, for replication. AAV is a human virus, with the majority of the population being seropositive for AAV, but no pathology has been associated with it. The virus contains two genes, *rep* and *cap*, encoding polypeptides important for replication and encapsidation, respectively (Fig. 4). These two genes can be supplied *in trans* with only the inverted terminal repeats (ITRs) required *in cis* for viral replication. Therapeutic genes with the appropriate regulatory sequences can be inserted between the two ITRs, and the virus generated by cotransfection into the 293 cell line with a *rep* and *cap* expression vector and subsequent infection with a first-generation adenoviral vector[44]. The wild-type virus can be grown to high titres and is able to integrate stably into a specific region of chromosome 19 following infection[45]. However, the recombinant virus appears not to integrate site-specifically, suggesting that this integration requires the presence of the rep protein.

One of the rate-limiting steps in AAV infection appears to be the frequency of second-strand synthesis[46]. In wild-type virus infection, second-strand synthesis is stimulated by the presence of adenovirus E1 and E4 proteins; in the absence of adenovirus coinfection, unknown cellular factors appear to dictate the rate of second-strand synthesis. In certain cell types, or following treatment with DNA-damaging agents, the rate of second-strand synthesis is high[47–49]. Moreover, in several cell types, it appears that the frequency of viral integration is reduced.

Although AAV may not be suitable for gene transfer to all cell types, the degree of infection of muscle, brain and liver cells with recombinant virus is exceedingly high *in vivo*. In these cell types, stable infection and gene expression apparently occur independently of the helper virus. Injection of a β-galactosidase-containing AAV vector into muscle has resulted in β-galactosidase-positive myofibres for up to two years[50–52]. Similarly, the injection of virus into the brain also has resulted in long-term gene expression[53]. Thus, AAV may be highly suitable for the delivery of genes to specific target cells *in vivo*, without inducing an immune response to the infected cells.

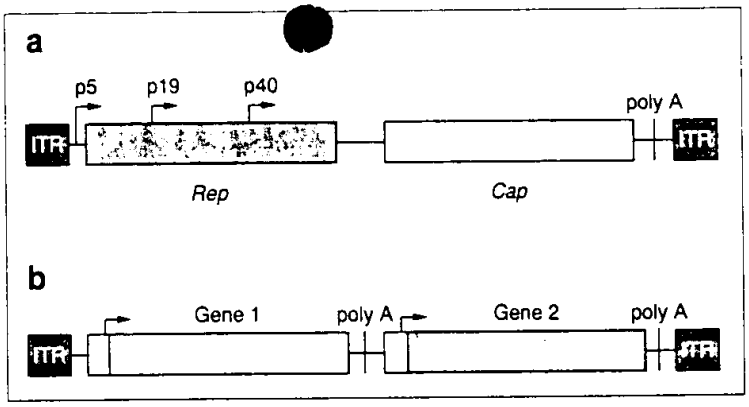

Figure 4

(**a**) Structure of adeno-associated virus (AAV). The position of the *rep* and *cap* genes, the inverted terminal repeats (ITR), the viral promoters (p5, p19 and p40) and the polyadenylation site (poly A) are indicated. (**b**) AAV can be used as a vector by inserting therapeutic genes between the ITRs.

Chimeric viral vectors

One of the future directions of vector development for gene therapy is the generation of chimeric vectors, which will have certain features of two or more viruses. For example, either a herpes-simplex virus or an adenovirus could be used to deliver AAV to cells in which transient expression of *rep* would allow site-specific integration of the AAV vector[54]. In this way, a large gene could be inserted into AAV. Alternatively, both the origin of replication and the *trans*-acting replication protein E1 from human papilloma virus could be inserted into a recombinant adenovirus vector; following infection, the episomal genome might be replicated and maintained by the HPV sequences. Similarly, HSV or adenovirus vectors could be used to deliver plus-strand RNA viruses, such as Sindbis, to cells[55]. Such viruses that have had their virulent capsid genes deleted could still replicate their RNA in the cytoplasm, allowing high levels of protein production[55].

Summary

Gene therapy is currently being applied to a wide range of diseases, both inherited and acquired. One, if not the, rate-limiting step to successful gene therapy is the efficiency of gene transfer. Currently, several different viral systems are being modified for use as vectors for gene therapy, including retroviruses, adenoviruses, herpes-simplex viruses, adeno-associated viruses and chimeric viruses. These viral vector systems

are currently in or approaching clinical trials, and they appear to be safe and well tolerated. Although it is likely that no single virus will be useful for all gene-therapeutic applications, with further development, viral vectors should become usable in specific gene-therapeutic applications.

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Diabetologia (1998) 41: 736–739

- Single cells.
- no functional studies
- beta cell specific.

Diabetologia

Transduction of non-dividing adult human pancreatic beta cells by an integrating lentiviral vector

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Summary Pancreatic islet cells are terminally differentiated endocrine cells and are refractory to stable infection by retroviral vectors, which require the breakdown of the nuclear membrane during cell division in order to insert the transgene into the host cell genome. Thus, attempts to render beta-cell allografts less immunogenic have had to rely on stable transfection of surrogate cells. Similarly, this problem has precluded the development of conditionally immortalized human beta cells for clinical allotransplantation. In this report, we demonstrate that adult human islet beta cells can be transduced by a new three-plasmid integrating lentiviral vector with an efficiency of 62 ± 1.8% at a multiplicity of infection (MOI) of 2.5 in vitro. This work makes genetic engineering of adult human pancreatic beta cells possible for the first time, allowing strategies to render beta-cell allografts non-immunogenic to be optimized and to creating conditionally immortalized human beta cells for clinical transplantation. [Diabetalogia (1998) 41: 736–739]

Keywords Lentiviral vector, retrovirus, human islet beta-cell, gene transfer, transplantation.

A major long-term goal of diabetes research is to re-establish normal glucose homeostasis in insulin-deficient patients by transplantation of insulin-producing beta cells [1]. The major unsolved problems of human beta-cell transplantation are overcoming allorejection, preventing recurrent autoimmunity, and expanding the limited supply of human tissue. Various approaches to these problems have shown promise in transgenic mice, but translation to adult human beta cells has been precluded because these terminally differentiated cells are refractory to stable infection by retroviral vectors [2], which require proliferation of the target cells and breakdown of the nuclear membrane during cell division in order to insert the transgene into the host cell genome. Thus, attempts to render beta-cell allografts less immunogenic have had to rely on stable transfection of surrogate cells. Similarly, this problem has precluded the development of conditionally immortalized human beta cells for functional study and clinical allotransplantation [3]

Recently, a new lentiviral vector system has been developed, based on the finding that human immunodeficiency virus (HIV) and other lentiviruses can infect nondividing cells [4]. It has been shown that this new vector system is able to efficiently and stably transfer genes into adult rodent brain and eye in vivo [5, 6]. In this report, we demonstrate the first successful transduction of adult human pancreatic islet beta cells, using lenti green fluorescent protein (GFP) and lenti-E. coli beta galactosidase gene (lacZ) vectors [4, 5].

Received: 26 January 1998 and in revised form: 17 March 1998

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Abbreviations: GFP, Green Fluorescent protein; lacZ, E. coli beta galactosidase gene; FCS, fetal calf serum; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate conjugate; CMV, cytomegalo virus; CRIP/MFG – cell packaging LINE, retroviral vector packaging cell line; MOI, multiplicity of infection.

Materials and methods

Preparation of human pancreatic islets. Human adult pancreatic islets were prepared at the Islet Transplantation Facility at the University of Giessen, Germany. Pancreata were obtained

within 8 h of death and islets were prepared as described previously [7]. In brief, following hypothermic perfusion via the abdominal aorta, organs were dissected in situ. After collagenase digestion, pancreata were digested in a continuous digestion-filtration device. Free islets were separated from nonislet tissue using a continuous Ficoll-Na-diatrizoate density gradient on a Cobe 2991 [7]. Islet purity of the preparations generally exceeded 80%. Single islets were suspended in Connaught Medical Research Laboratory (CMRL)-1066 medium containing 10% fetal calf serum (FCS), 1% glutamine, 1% N-2-hydroxyethyl piperagine N'-2 ethane sulphonic acid (HEPES), 1% Pen/Strep, and incubated at 25 °C. The media were changed every week. The islet preparation was examined visually for bacterial or fungal contamination. Dithizone (0.02% for 3 min) was used to detect contamination by non-endocrine cells (data not shown).

Preparation of human islet cells. To prepare single islet cells, approximately 5000 islets were digested in 8 ml of calcium-free phosphate buffered saline (PBS) containing 0.125 mg/ml of trypsin and 0.05 mg/ml ethylenediaminetetraacetic acid (EDTA) at 37 °C. The islet solution was rotated for 5 min at 37 °C and then placed on ice for 5 min to allow islets to settle. The supernatant containing the single islet cells was removed, 1 ml of FCS was added and the solution centrifuged in Connaught Medical Research Laboratory media for counting. The digestion cycle was repeated a maximum of four times to obtain additional cells.

Characterization of adult human islet cells. The purity of the isolated human islet cells was analysed by immunofluorescent staining using a guinea pig anti-porcine insulin antibody which is cross reactive against human insulin (Chemicon International, Temecula, Calif., USA). 10^5 cells per well were plated in 24 well plates the day before immunofluorescent staining. The cells were washed briefly with PBS, fixed in cold 4% paraformaldehyde for 15 min, washed with PBS, and blocked with 10% FCS/10% goat serum/0.2% triton in PBS for 30 min. Primary antibody, guinea pig anti-porcine insulin, (diluted 1:100) was added and the plate was incubated for 1 h. After washing with PBS, second antibody, fluroescein isothiocyanate conjugate (FITC) conjugated donkey anti-guinea pig IgG (diluted 1:100) was added and the plate was incubated for 1 h in the dark. After washing with PBS, the plate was examined using fluorescence microscopy.

Preparation of lentivirus. 293T cells were plated in 100 mm cell culture plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 1% Pen/Strep, and grown to 70% confluency. A total of 40 µg of plasmid DNA was used for the transfection in the following proportions using Perfect Lipids no. 2 (Invitrogen Carlsbad Calif USA) according to the manufacturer's instructions: on to megato virus cmv abbreviation in 15 µg of pCMVΔR8.2, 20 µg of pHR'CMV-GFP or pHR'CMV-LacZ and 5 µg of pMD.G as described [4, 5]. These plasmids are gifts of Dr. D. Trono at the University of Geneva, Switzerland. Conditioned medium containing lenti-GFP or lenti-lacZ viruses were harvested 48 to 60 h after transfection, subjected to low-speed centrifugation, and filtered through 0.45 µmol/l filters. Viruses were further concentrated by ultracentrafugation as described. The viral preparation were frozen at –80 °C. For lenti-GFP, the 293T cells left on the plate were examined under fluorescence microscopy for percentage of green cells. For lenti-LacZ, the 293T cells left on the plate were fixed with 1% glutaraldehyde and stained with X-gal (see below). More than 80% of the cells turned green (lenti-GFP) or blue (lenti-LacZ) indicating that the cotransfection of the three plasmids was successful. To titrate the viral particles in the conditioned medium, 5×10^5 of 293T cells per well were infected overnight at 37 °C in six-well plates with serial dilutions of conditioned medium supplemented with polybrene (8 µg/ml]. The medium was replaced, the cells further incubated for 36 h, and expression of GFP or β-galactosidase scored by fluorescence microscopy or X-gal staining. Titres were calculated by counting the number of the foci of green or blue cells per well and dividing the number by the dilution factor.

Preparation of retrovirus. Retroviral producer cells, retroviral vector packaging cell line-ecoli beta galactosidase gene (CRIP/MFG-LacZ), were from Dr. R.C. Mulligan. CRIP/MFG-LacZ cells were grown to confluency as described and the supernatants containing MFG-LacZ viral particles were centrifuged at low speed, filtered through a 0.45 µmol/l filter and frozen at –80 °C. 293T cells were used to titre each preparation as described above.

Infection of human islet cells with lentiviral and retroviral particles · 10^6 single adult human islet cells were plated in 60 mm polylysine-coated plates 2 days before infection. Viruses were added to the CMRL-1066 medium supplemented with polybrene (8 µg/ml) at different multiplicity of infection (MOIs) and the cells were infected overnight. The media was replaced and the cells further incubated at 37 °C. Expression of the GFP reporter gene was examined 5 days after infection. Cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature and washed again with PBS. Fixed cells were fluorescence-immunostained using guinea pig anti-porcine insulin antibody (Chemicon International, Temecula, Calif., USA) as the first antibody and Rhodamine-Red conjugated donkey anti-guinea pig IgG (Jackson Immuno Research West Grove PA USA) as the second antibody. Uninfected islet cells in separate plates were fixed and stained under the same conditions, and used as negative controls. Green cells and red cells of the same field were photographed using a fluorescence microscope. Two images (green and red) were scanned using Adobe Photoship Programme (Apple Computer B cuper + INw, Calif USA) and were then matched to give the double exposure image. Expression of the β-galactosidase was scored by X-Gal staining. The cells were washed twice with PBS, fixed with 1% glutaraldehyde at room temperature for 5 min, washed again with PBS and stained with X-gal/Fe solution for 4 h at 37 °C. Uninfected islet cells in separate plates were fixed and stained under the same conditions, and used as negative controls. To avoid background of the endogenous β-galactosidase of islet β-cells, X-gal staining was done for 4 h.

Quantitation of infection efficiency and statistical methods. Data of infection efficiency were analysed using the descriptive statistics analysis software of Microsoft Excel.

Results

Pancreatic islets were prepared from human pancreas using Ricordi's automated method [7]. Since pancreatic islets contain four major cell types, alpha, beta, delta and pancreatic polypeptide, the purity of islet preparations was determined before viral infection. Single cells were prepared from human islets and plated onto cell culture dishes. An immunofluorescence assay using antibody against porcine insulin was used

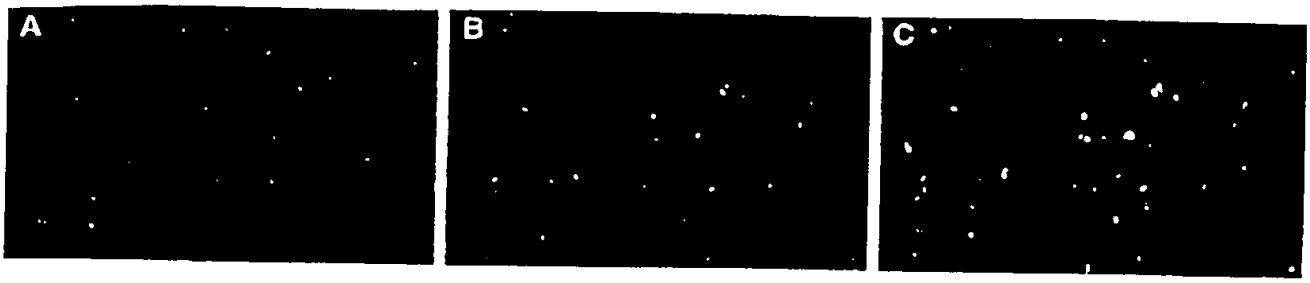

Fig. 1. Single human islet cells plated in polylysine-coated plates were infected with lenti-GFP viruses at a MOI of 2.5 in the CMRL-1066 medium supplemented with polybrene overnight. Five days after infection, cells were washed with PBS, fixed with 4% paraformaldehyde and immunostained using guinea pig anti-porcine insulin antibody and Rhodamine-Red conjugated donkey anti-guinea pig IgG. Red cells (A) and green cells (B) of the same field were photographed using a fluorescence microscope (100x). Two images (green and red) were scanned using Adobe Photoship Program and were then matched to give the double exposure image (C). **A**, human islet β-cells (red) positive to insulin antibody. **B**, human islet cells (green) infected by lenti-GFP vectors. **C**, human islet β-cells infected by lenti-GFP vectors (orange)

to identify the major cell type of these single cells. After immunostaining, the number of total cells and the number of insulin-producing cells were counted under the fluorescence microscope. 864 cells in 11 groups were counted and the average percentage of insulin-producing beta-cells was obtained. The results indicated that $82 \pm 2.5\%$ of the single cells prepared from human islets were insulin-producing beta-cells.

The transducing capacity of the lentiviral preparation in human islet beta-cells was first assayed quantitatively using lenti-GFP virus [5]. Single islet cells were prepared from human islets and plated in polylysine-coated 6 or 12-well plates and infected overnight with lenti-GFP viral particles at a MOI of 2.5. The infected human islet cells were examined under the fluorescence microscope every day. Green cells started to appear 2 days after infection. Five days after infection, the number of green cells reached its maximum. 909 cells in seven groups were counted and the average percentage of green (infected) islet cells was $68 \pm 2.6\%$ (data not shown). In order to quantitate the infection efficiency of beta-cells, cells were fixed with 4% paraformaldehyde and analysed by immunofluorescent assay using a guinea pig anti-porcine insulin antibody which is cross reactive against human insulin. Rhodamine-Red conjugated donkey anti-guinea pig IgG was used as the second antibody. The plate was examined using fluorescence microscopy for red (beta-cell) and green (lenti-GFP infected) cells, and photographed. If one cell was both red and green, it was a lenti-GFP infected beta-cell. 360 cells were counted in six separate fields. As illustrated in Figure 1, $62 \pm 1.8\%$ of the beta-cells (red cells in Fig. A) were transduced by lenti-GFP viral particles and expressed green fluorescent protein (green cells in Fig. B). Double exposure rendered lenti-GFP infected beta-cells orange and clearly distinguishable from the uninfected beta-cells (Fig. 1C). Since integration is a necessary step for the expression of transgenes delivered by both retroviral and lentiviral vectors [4], these results indicated that the lentiviral particles could deliver and integrate the GFP reporter gene into the genome of adult human beta-cells efficiently.

Although beta-cells are differentiated endocrine cells and do not proliferate in culture, a recent study on beta-cell regeneration suggested that differentiated beta-cells still possess the potential to replicate and divide under certain conditions [8]. To exclude the possibility that those infected beta-cells were dividing at the time of infection, human islet cells were then infected with a retroviral vector which only transduces dividing cells and the results were compared with that of the lentiviral vector which transduces both dividing and non-dividing cells. MFG-LacZ viral particles were prepared from retroviral producer cells, CRIP/MFG-LacZ (a gift from Dr. R. C. Mulligan). 293T cells were used as a control of dividing cells in this experiment. 293T cells and single human islet cells were infected with lentiviruses and retroviruses separately with the same MOI of 2.5. 293T cells were stained with X-gal 3 days after infection and the human islet cells were stained after 5 days. Uninfected cells were stained as negative controls. Figure 2 shows that lentivirus can transduce both 293T cells (dividing cells) and isolated human islet cells (non-dividing cells) and retrovirus can only transduce dividing 293T cells. The results clearly indicate that the human islet beta-cells infected by lentiviruses were non-dividing cells.

Discussion

Our long-term goal is to establish conditionally immortalized, non-immunogenic human islet beta-cell lines that can ultimately be used for transplantation therapy of diabetes mellitus. As an initial step towards this goal, we have shown that a lentiviral expression system successfully delivered the GFP and LacZ reporter genes into single non-dividing human

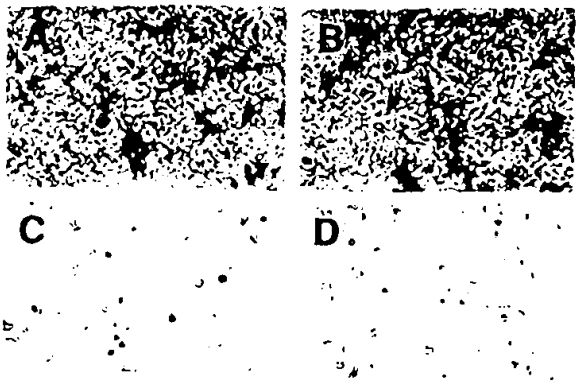

Fig. 2. Viruses were prepared and titrated. Single human islet cells (C,D) and 293T cells (A,B) plated in polylysine-coated 6-well plate were infected overnight with viruses (A and C for lenti-lacZ, B and D for retro-lacZ) at MOI of 2.5 in CMRL-1066 medium supplemented with polybrene (8 μg/ml). The medium was replaced, the cells further incubated, and expression of β-galactosidase scored by X-Gal staining 5 days after infection. Cells were photographed with a research microscope under phase-contrast. Different groups of cells are shown in each photograph (100×). A, 293T cells infected with lentiviral-lacZ. B, 293T cells infected with retroviral-lacZ. C, human islet cells infected with lentiviral-lacZ. D, human islet cells infected with retroviral-lacZ

islet beta-cells in vitro. Since it has been well established in retrovirus that integration is an obligatory step for retroviral gene expression [4], our results also demonstrate that the delivered reporter genes were integrated into the genome of adult human islet beta-cells.

This is the first time that adult human islet beta cells have been successfully transduced with an integrating vector. Fetal human islet cells have been successfully transduced with retroviral vectors, since these immature islet cells are still proliferating [9]. However, the incomplete differentiation and extremely limited availability of these cells make their use impractical for generating sufficient quantities of genetically engineered beta cells for ultimate clinical application.

The integrating three-plasmid lentiviral system shown in this report to transduce adult human beta cells has been shown previously to transfer genes into adult rodent eye and brain in vivo [6,10]. However, since genes deleted from lentiviral vectors may be superfluous for nuclear transport of the preintegration complex in some cell types, but essential in others, the ability of such vectors to transduce a given cell type must be empirically determined for each cell type. Similarly, the duration of expression of such vectors must be individually assessed in each tissue type. The expression of the lentivirus vector has been detected in neurons for up to 6 months [5]. This suggests that unlike retroviral vectors whose expression is silenced due to integration into a region of the host genome where transcription is downregulated [11], integrating lentiviral vectors may overcome the problem of gene silencing by integrating into stably open chromatin. The duration of transgene expression in adult human beta cells transduced with this vector remains to be determined and cannot be assumed a priori.

The two major obstacles to using adult human beta cells as transplantation therapy for diabetes mellitus are (a) the need to avoid the use of toxic immunosuppressive drugs to prevent allograft rejection (and in Type 1 patients, autoimmune disease recurrence), and (b) the need to provide much larger amounts of donor human tissue than are currently available from cadaveric sources [3]. The data obtained in this study demonstrate that adult human beta cells can now be successfully transduced with integrating lentiviral vectors, making it possible for the first time to optimize genetic engineering strategies to render beta-cell allografts non-immunogenic and to create stable, conditionally immortalized beta-cell lines for eventual clinical application.

Acknowledgements. We would like to thank Dr.Didier Trono at the University of Geneva, Switzerland for kindly providing the three plasmid lentiviral expression systems. This work was supported in part by Interdisciplinary Program Project grant no. 892001 from the Juvenile Diabetes Foundation International.

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long-term survivors initially pass through a reactive phase against donor antigen, and that this reactivity lasted several months, but eventually subsided. Thus some form of peripheral T cell inactivation must also play a role in the eventual development of tolerance. Similar nonreactivity following antigen administration in a rat model has been described and attributed to peripheral anergy (*17*).

Although the bone marrow inoculum was shown to be essential for establishing multilineage mixed chimerism in this protocol, it seems likely that the surviving kidney allograft may also be involved in the maintenance of tolerance. It has been suggested by Starzl and colleagues (*18*) that long-term acceptance of allografts in patients may always involve the establishment of a state of microchimerism in which the allograft itself is the source of the chimeric population. In our previous murine models in which mixed chimerism persisted indefinitely (*13*), there was no need to invoke an organ or graft as responsible for maintaining tolerance. Indeed, graft could be placed on these animals and be accepted y time for the remainder of their lives. Studies are in progress in our monkey model to determine whether or not the kidney allograft is required for tolerance induction using the present preparative regimen. Additional studies are directed toward better T cell depletion in an attempt to achieve ore stable mixed chimerism without the need for CsA, and ward potential use of radiomimetic drugs rather than irradiation in an attempt to reduce further the morbidity of the reparative regimen. Finally, although the timing of irradiation and host T cell depletion used here might be applicable living allogeneic donors, both treatments could not start ore than 24 hr prior to transplantation for cadaver donor ansplants. Current studies are therefore directed toward trying the timing of T cell depletion and irradiation with spect to the kidney transplant in an attempt to make the otocol more clinically applicable.

Acknowledgments. The authors thank Dr. Siew Lin Wee for advice performance of in vitro studies; Ms. Catherine Padyk, M.B.A. for pert editorial assistance; and Dr. Han Zhou Hong, Dr. Svjetlan oskovic, and Mrs. Patricia Della Pelle for technical assistance.

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Received 1 June 1994.
Accepted 30 August 1994.

0041-1337/95/5902-263$03.00/0
TRANSPLANTATION
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Vol. 59, 263–268, No. 2, January 27, 1995
Printed in U.S.A.

EFFICIENT GENE TRANSFER TO PANCREATIC ISLETS MEDIATED BY ADENOVIRAL VECTORS[1,2]

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Genetic manipulation of pancreatic islets before transplantation has the potential to alter cellular immunity as well as islet function. The purpose of this study was to examine the feasibility of gene transfer to islets, using replication-defective adenoviral vectors. Newborn mouse islets were infected with AdHCMVsp1LacZ vector encoding *Escherichia coli* β-galactosidase (β-gal). Islets were cocultured with vector, at virus-to-target cell ratios of 10:1, for 1 hr. Gene transfer was assessed by specific histochemical stain for β-gal (X-gal). Islet DNA and RNA were analyzed by Southern and PCR for β-gal and adeno sequences, and recombinant protein production by western and ONPG assays. Islet integrity after gene transfer was assessed by static incubations and transplantation to nondiabetic and to diabetic mice. Southern analysis and PCR confirmed the presence of *E coli* β-galactosidase and the E4 adeno DNA in infected islets, but not in controls. Reverse-transcription PCR and western analysis demonstrated expression and protein production of inserted *E coli* β-galactosidase, but not E4 message. Insulin release in response to static incubations was unimpaired in infected islets. Syngeneic islet grafts stained positively for insulin for up to 7 days. Transplanted, genetically manipulated islets functioned similarly to control islets in reversing murine drug-induced diabetes. Thus, gene transfer into islets can be accomplished using adenovirus-based vectors. The capacity of this virus to infect nondividing cells allows insertion of cDNA into pancreatic islets, with potential application to the transplant setting.

Treatment of type 1 diabetes with transplantation of pancreatic islet cells results in insulin-independence in only a minority of transplant recipients, as islets are subject both to allograft rejection (*1*) and to autoimmune destruction (*2*). Gene therapy represents one potential intervention to enhance survival of these grafts.

Transfer of genetic sequences with the potential to downregulate the local immune responses may diminish both rejection and autoimmune-mediated graft death. Several options for gene transfer to somatic cells have been described, including direct DNA precipitation as well as viral-mediated transfer technologies (*3*, *4*). Replication-defective retroviruses have been most extensively described as shuttle vectors, but their efficacy is limited to actively replicating target cells, precluding their use in cultured islets (*5*). In contrast, replication-deficient adenoviral vectors do not require target cell replication, are tropic to a wide variety of mammalian cell types, and therefore have potential utility in islet-directed gene transfer (*6*, *7*). In vitro adenoviral-mediated gene transfer has been described in hepatocytes and endothelial cells, yielding successful expression and protein production of inserted genetic sequences (*8*, *9*). This project, then, was designed to define reproducible conditions for gene transfer in cultured newborn mouse islets using a replication-deficient adenoviral vector encoding the reporter gene, *E coli* β-galactosidase (β-gal).* Islet integrity after gene transfer was assessed by static incubation assays for insulin release. Infected murine islets were transplanted in a syngeneic model in order to document the functional life span of viral-derived genes. To further test islet integrity, genetically manipulated islets were grafted under the kidney capsule of mice rendered diabetic by streptozotocin. Nonmanipulated islets and controls were similarly transplanted. Results obtained in this study demonstrate that replication-deficient adenoviruses are effective mediators of gene transfer to cultured islets. Furthermore, islet integrity is preserved after adenoviral-mediated gene transfer. The use of this model with a viral vector encoding proteins capable of inhibition of alloreactive immune responses has potential therapeutic applicability for enhancing islet survival after transplantation.

MATERIALS AND METHODS

Islet isolation. Under general anesthesia, pancreata were removed from 2–10-day-old BALB/c mice using sterile technique. The organs were sectioned into 1–2 mm^3 pieces, digested in collagenase P (Boehringer-Mannheim) 1.5 mg/ml, in a shaker bath at 37°C. In general, islets were freed within 5 min, as assessed by dithizone staining of aliquots. Islet suspensions were washed three times in HBSS for 5 min at 1000 rpm at 4°C, plated in RPMI + 10% NuSera + 5% fetal bovine serum (fbs) + 2.5% Hepes buffer, pH 7.27, and cultured at 37°C. Medium was replenished a day later, and every 48 hr thereafter.

Viral vectors. The adenoviral vector, AdHCMVsp1LacZ, derived from adenovirus serotype 5 backbone (*10*) contains an expression

[1] Presented at the 20th Annual Meeting of the American Society of Transplant Surgeons, May 18–20, 1994, Chicago, IL.

[2] Supported by Grant 193115 (M.E.C.) from the Juvenile Diabetes Foundation International (The Children's Diabetes Foundation at Denver Research Grant).

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* Abbreviations: β-gal, beta-galactosidase; MOI, multiplicity of infection; ONPG, o-nitrophenyl-β-D-galactopyranoside; pfu, plaque-forming units; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-indolyl-β-D-galactopyranoside.

cassette with the LacZ gene (encoding β-gal), under control of a CMV promoter and upstream of a polyadenylation signal. The expression cassette replaces the entire viral E1A (early viral protein) region and most of the E1B region, rendering the virus replication-defective. The virus is able to replicate only in 293 kidney embryonal cells that constitutively express the E1 proteins. To generate high titer viral stocks, 293 cells were infected at a 5:1 plaque-forming unit (pfu):293 ratio. Infected 293 cells were harvested after 48 hr, and virus was recovered using cycles of freeze-thawing. Viral titers were assessed by plaque assay. Viral stocks of 10^{10} pfu/ml were stored at −80°C.

Infection methods. Freshly isolated or cultured islets (after removal by gentle pipetting with Ca^{++}-Mg^{++}-free HBSS) were counted then allowed to pellet at room temperature in minimal volume of medium. Each islet was estimated to contain 1000 cells. AdHCMVsp1LacZ was added to the pellet at a multiplicity of infection (MOI) of 10 pfu/target cell for 1 hr at room temperature. Islets were washed, then recultured at 37°C until assayed.

X-gal staining. Gene transfer was assessed by direct histochemical staining for β-galactosidase protein, 36 hr after infection and periodically thereafter. Islets were first fixed in 1.25% glutaraldehyde at 4°C for 10 min, rinsed, then immersed in 5-bromo-4-chloro-indolyl-β-[redacted]pyranoside (X-gal). Cells expressing β-galactosidase turn in the presence of X-gal, while nonexpressing cells appear clear to faintly yellow. The percentage of cells within the islet staining blue yields an estimation of the efficiency of gene transfer. Uninfected islets from the same donors were stained as negative controls. In order to further assess efficiency of gene transfer, infected islets were dispersed (*11*) after X-gal staining, and individual cells scored for staining using a hemacytometer.

DNA polymerase chain reaction (PCR) and Southern analysis. Islets were rinsed in iced RNAse-free saline, and frozen immediately in liquid nitrogen. Islet DNA (genomic and episomal) was purified using phenol-chloroform extraction, followed by suspension in TE buffer. A positive control consisted of DNA extracted from AdHCMVsp1LacZ-producing 293 cells. DNA 1 μg was amplified by PCR in a total volume of 50 μl containing 0.2 mM deoxynucleoside triphosphate, 0.25 μM of 5′ and 3′ oligonucleotide primers, 50 mM KCl, 5 mM Tris-Cl (pH 8.3), 1.5 mM $MgCl_2$, and 2.5 U Taq polymerase (Perkin-Elmer, Norwalk, CT). The oligomeric primers flank an internal 1036 bp sequence of the LacZ gene for demonstration of transferred sequences (primer 1: GCCGACCGCACGCCGCATCCAGC, primer 2: CGCCGCGCCACTGGTGTGGGCC). Flanking primers were designed to detect a 535 bp DNA sequence from the adenovirus E4 region (primer 1: CGCAGGGCCAGCTGAAC, primer 2: CCGAAATAGCCCGGGGG), downstream from the LacZ gene. PCR was performed in a DNA thermal cycler (Perkin-Elmer). The amplification profile consisted of 30 cycles of denaturing for 1 min, annealing of primers at 60°C for 2 min, and extension at 72°C for 2 min. PCR products obtained in this manner were analyzed by gel electrophoresis. In parallel, Southern analysis was performed using probes hybridizing LacZ and E4 sequences.

Reverse-transcription PCR for RNA analysis. RNA was extracted using a standard RNAzol method (*12*). AdHCMVsp1LacZ-producing 2[redacted] were again used as positive controls. RNA was added to a solution consisting of 2 mM $MgCl_2$, 1× PCR buffer II, 1 mM deoxynucleoside triphosphate, 1 U/μl RNase inhibitor, 2.5 U/μl reverse transcriptase, and appropriate primers (Random Hexamers, Oligo d(T), downstream primers). The solution was overlaid with 50 μl mineral oil, and incubated at room temperature for 10 min. The amplification cycle profile consisted of 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min for one cycle, then the DNA PCR thermal protocol was used for 30 cycles.

Western blots. For protein extraction, islets were frozen in liquid nitrogen, fractured, and vortexed in 500 λ NTM buffer (0.01 M Tris pH 7.6, 0.21 M NaCl, 0.1 M MgOAc, 0.1 M β-mercaptoethanol, 0.001 M EDTA). Sample protein concentrations were measured spectrophotometrically. Protein 2.5 mg was separated in 10% SDS-PAGE, and electrically transferred to nitrocellulose. After blocking for nonspecific binding with 1% BSA, the membrane was incubated with a primary anti-E coli β-gal monoclonal antibody (Sigma), washed, and incubated with a secondary antibody-horseradish peroxidase conjugate (Organon Teknika). Detection of the recombinant protein band was accomplished by chemiluminescence. For relative quantification, study columns were compared with known standards of recombinant *E coli* β-galactosidase protein (Sigma) prepared in the same fashion.

O-nitrophenyl-β-D-galactopyranoside (ONPG) assay. For protein extraction, islets were frozen in liquid nitrogen, fractured, and vortexed in 0.21 M NaCl, 0.10 M MgOAc, 0.01 β-mercaptoethanol, and 0.001 M EDTA. Total islet protein concentrations were calculated spectrophotometrically using a Bradford assay. β-galactosidase concentration was determined using the chromogen ONPG as a substrate for enzymatic activity. An aliquot of total islet protein was incubated in ONPG solution (*13*) at 37°C for 30 min and the reaction was halted by addition of Na_2CO_3. Optical density of the solution was read at 420 nm, and compared with a standard curve generated using known quantities of recombinant β-galactosidase. Sample β-galactosidase concentrations were recorded as a percentage of total cellular protein concentration.

Static incubation insulin release assay. At 48 hr after gene transfer, islets were incubated in 2.8 mM (low) glucose RPMI + 10% fetal calf serum for 18 hr. Supernatant was collected for baseline insulin release (assayed later using standard radioimmunoassay) after a 45-min incubation in fresh, low glucose medium. The islets were then incubated in 16.7 mM (high) glucose for 45 min, followed by low glucose for 45 min, then high glucose with 10 mM theophylline for 20 min. All incubations were followed by extensive washes after collection of supernatant for insulin assays. These results were normalized to islet equivalents. Stimulation indices were then calculated by dividing the insulin response to high or high + theophylline incubations by the response to low glucose. Control islets were batch-matched islets not exposed to adenovector. Islets were counted, and viability assessed at the beginning and end of the assay using exclusion of trypan blue. All assays were performed in triplicate.

Transplantation of infected islets. Under general anesthesia, 200 syngeneic newborn BALB/c control (noninfected) and infected islets (two days after infection with replication-deficient adenovirus vectors), were transplanted under the left kidney capsule of adult BALB/c mice. The kidneys were removed at days 1–7, embedded and frozen, then later stained for the viral gene product with X-gal.

Insulin staining of transplanted islets. Paraffin sections of kidney sections were dewaxed with xylene then ethyl alcohol, then stained for insulin using a double-antibody technique. The primary antibody was guinea pig antiinsulin (1:500), and the secondary antibody was biotinylated rabbit antiguot (1:200). The slides were exposed to alkaline phosphatase, then fast red, and counterstained with hematoxylin. Insulin-expressing cells appeared red under light microscopy.

Islet transplantation in diabetic mice. BALB/c mice were injected in a tail vein with 160 mg streptozotocin, and blood glucose levels followed periodically thereafter. When two consecutive blood glucose readings exceeded 300 mg/dl, the mice were considered diabetic. At that time, the mice received syngeneic islet grafts under the left kidney capsule as described above. Half the mice received islets 24–48 hr after gene transfer with AdHCMVsp1LacZ, and half the mice received batch-matched islets not exposed to the viral vector. Blood sugars were followed twice weekly. After about one month the left kidney was removed, and blood sugars were followed serially.

RESULTS

X-gal staining. All islets in infected samples evidenced some blue cells in the islet cluster, with considerable variability in the number of cells infected per islet and in the intensity of staining. In some islet clusters only a single stained cell was apparent, whereas in other clusters all cells appeared blue (Fig. 1A). Control, noninfected islets from the same samples did not stain positively with X-gal (Fig. 1B). The difference between infected and control cells was unambiguous with X-gal incubation times from 15 min to 2 hr. Prolonged exposure to the staining solution resulted in some staining of noninfected cells, presumably due to endogenous β-gal activity. This pattern was maintained for up to 10 days in culture after gene transfer, suggesting relative stability and expression of the transferred gene.

Infected islets were stained with X-gal then dispersed in order to more accurately count the number of individual cells turning blue. The intensity of blue staining varied greatly from cell to cell—however, at aMOI of 10:1, 27% of these islet cells clearly stained blue with X-gal.

DNA PCR, Southern analysis, and RNA PCR. Using PCR, DNA extracted from infected islets demonstrated LacZ sequences, as did the positive adenovirus-producing 293 cells. A band corresponding to the sequence between the LacZ oligoprimers was seen at 1036 bp (Fig. 2A). No such band was demonstrable in DNA extracted from uninfected islets. These results were confirmed using Southern analysis with probes specific for LacZ and E4 sequences. Reverse-transcription PCR demonstrated *E coli* LacZ mRNA in infected islets only (Fig. 2B). No message for LacZ sequences was demonstrable in RNA extracted from uninfected islets. Furthermore, RNA PCR revealed no message for adenoviral E4, confirming the lack of (wild-type) viral replication. These results confirm uptake of the replication-deficient viral vector by the islets, and successful gene transcription of the virally encoded LacZ sequences.

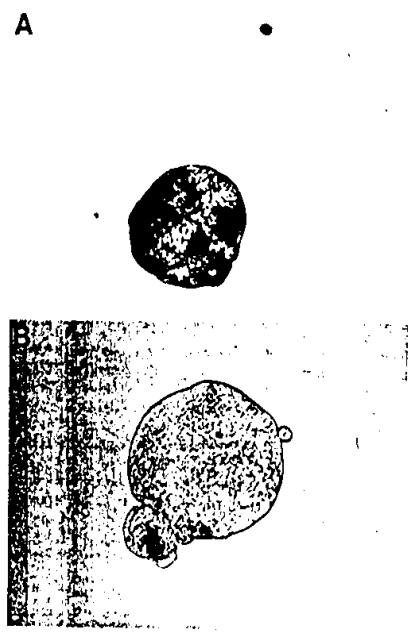

FIGURE 1. Successful gene transfer to islets was demonstrated by X-gal staining. In some islets the majority of cells expressed the gene product, as evidenced by dark blue staining (A). Control, uninfected islets did not stain with X-gal (B).

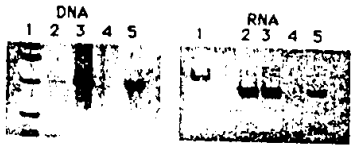

FIGURE 2. (Left) Islet DNA was examined by PCR using synthetic oligomers with sequences specific for *E coli* β-galactosidase. The inserted LacZ (β-gal) gene was detected with a band at 1036 bp in the viral packaging cell line (lanes 2, 3) and in infected islets (lane 5), but not in uninfected islets (lane 4). (Right) Similarly, reverse PCR demonstrated β-gal message in the packaging cell line (lanes 2, 3), and infected islets (lane 5), but not in uninfected islets (lane 4). DNA ladder marker was run in lane 1 in both preparations.

Western blots. Using western blotting, specific quantities of cytoplasmic protein extracts of infected and control islets were incubated with anti-*E coli* β-gal mAb. An induced protein corresponding to β-gal was clearly identified at a MW of 110 kD; β-gal protein was not found in uninfected islet cell extracts. (Fig. 3).

ONPG assay. In order to further quantify protein production, and therefore gene transfer efficiency, this functional assay was performed on islet batches exposed to varying MOI. At aMOI of 1:1, ONPG assay revealed that β-gal accounted for 0.9% of total islet protein. At a MOI of 10:1, β-gal was 1.1% of total islet protein and at a MOI of 100:1, β-gal was 1.9% of total islet protein.

Static incubations. Stimulation indices, calculated by dividing the insulin response to high glucose by that to low glucose, or by dividing the response to high glucose + theophylline to low glucose are shown in Table 1. Insulin responses to high glucose and to high glucose plus theophylline were similar in control and infected islets, as assessed by stimulation indices. These results suggest that adenoviral-mediated gene transfer to islets does not destroy islet integrity.

Kidney histology and insulin staining of transplanted islets. Kidneys injected with infected islets were removed a week after subcapsular transplantation. X-gal staining of fro-

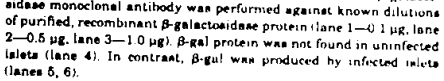

FIGURE 3. Western blot analysis using an anti-*E coli* β-galactosidase monoclonal antibody was performed against known dilutions of purified, recombinant β-galactosidase protein (lane 1—0.1 μg, lane 2—0.5 μg, lane 3—1.0 μg). β-gal protein was not found in uninfected islets (lane 4). In contrast, β-gal was produced by infected islets (lanes 5, 6).

TABLE 1. Stimulation indices of insulin production using static incubation*

	High low	High low	High + theophylline low
Control	1.6	1.5	
Infected	2.6	1.5	
Control		1.4	2.7
Infected		1.5	3.5
Control		1.9	7.5
Infected		2.1	9.5

* Stimulation indices were calculated by dividing the insulin response to high glucose (or high glucose + theophylline) incubation by the insulin response to low glucose incubation, in three separate experiments. The similar indices in infected and control islets indicate that gene transfer does not impair normal insulin responses to glucose and to theophylline challenges.

sections revealed stained clusters of islets adjacent to l kidney tissue. Paraffin sections of kidneys transed with infected and noninfected islets were stained for insulin 7 days after transplantation. Both groups stained positively for insulin (whereas normal kidney did not stain), and there was no apparent difference in the pattern of insulin staining between uninfected islets and those exposed to the vector.

Islet transplantation in diabetic mice. Blood glucose readings from a representative pair of transplanted mice are shown in Figure 4. After transplantation of 200 syngeneic islets, both mice with infected islets and noninfected islets maintained blood glucose levels of about 200 mg/dl over the course of one month. There was no difference in blood glucose

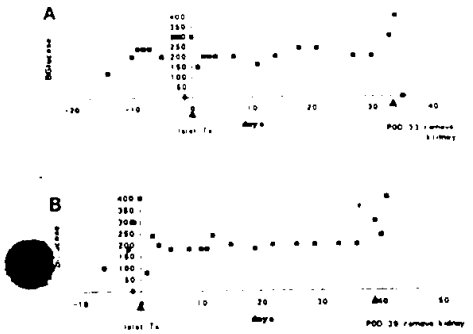

FIGURE 4. Blood glucose levels in response to islets transplanted under the left kidney capsule. Mice were injected with 160 mg streptozotocin i.v., and considered diabetic after two consecutive blood glucose readings above 300 mg/dl. At that time (day 0) the mice received 200 syngeneic islets, injected under the left kidney capsule. (A) A representative blood glucose pattern after transplantation of normal cultured newborn mouse islets. After transplantation, blood glucose readings were consistently about 200 mg/dl. (B) Islets transplanted 24 hr after gene transfer with AdHCMVsp1LacZ mediated stable blood glucose levels—not different from the control islets. In both, left nephrectomy about a month after islet grafting resulted in rapid return of pretransplant hyperglycemia.

levels between mice transplanted with islets after gene transfer and control islets. Removal of the left kidney also resulted in similar return of pretransplant hyperglycemia. Furthermore, X-gal staining of the nephrectomized grafts revealed blue clusters in the mouse transplanted with adenoviral-exposed islets, but not in controls. These results have been repeated with 3 other pairs. In all cases, blood glucose patterns were similar between pairs.

DISCUSSION

Pancreatic islet transplantation is an attractive theoretical option for the treatment of patients with type 1 diabetes. However, despite recent advances in the isolation (*14*), purification (*15*), and preservation (*16*) of human islets, clinical islet transplantation has not provided insulin-independence in the vast majority of recipients (*1*). Transplanted islets are the targets of both alloimmune and autoimmune-mediated destruction, as well as nonspecific inflammatory reactions (*17*) and site-specific chemical mediators (*18*).

Ex vivo manipulation of islets in culture prior to transplantation has improved islet graft survival in several experimental models (*19, 20*), suggesting that modification of the antigenic profile of the islets can impact survival. Therefore, methods of masking target cell antigenic expression or interfering with the host immune cascade directed at the islet are rational approaches to enhancing islet graft survival.

Gene transfer is an attractive option for modifying islet antigenicity prior to transplantation. Potential gene therapy approaches for altering the alloreactive immune response include induced expression of determinants recognized as self by the host, such as transfer of sequences expressing self-MHC antigens (*21, 22*). Another approach is to enhance expression of proteins capable of inhibiting the cell-mediated immune cascade, such as TGF-β or viral interleukin-10 (IL-10/BCRF) (*23, 24*).

The various technical methods for gene transfer to islets have not been explored. Retroviruses are well-characterized vectors for gene transfer in many replicating mammalian cells (*3, 5*). However, the quiescent nature of islets in culture limits the utility of retroviruses for efficient gene transfer in this system. Preliminary work in our laboratory, in which cultured islets were exposed to retroviral vectors encoding reporter genes, failed to produce detectable transduction (data not shown). In contrast, in our system, adenoviral vectors demonstrated efficient gene transfer to islets. The advantages of the adenoviral vector, then, include the ability to infect diverse mammalian cell types, independent of active target cell replication (*6, 7*). Furthermore, only short-term exposure to the virus is required for rapid uptake and expression in infected cells. This rapid uptake is most likely due to a high density of adenovirus receptor sites on mesenchymal cells (*25*). Though the density of adenovirus receptor sites on islets has not been characterized, the efficiency of adenoviral-mediated gene transfer in our model implies a clear tropism for cultured islets.

The tropism of the replication-defective adenovirus for cultured mouse islets was patently demonstrated by screening of islets infected with the virus and noninfected islets using X-gal staining. Following a 1-hr exposure of islets to virus, all islets contained blue-stained cells, with marked variability in positivity from islet to islet, but an average positivity rate of 27%. The high virus-derived β-gal activity in infected cells

resulted in appearance of blue cells a short time after incubation with stain. The rapidity of this response contrasted clearly with the nonspecific, relatively late endogenous β-gal staining in control islets. Gene transfer was confirmed by the presence of viral DNA in infected cultures, but not in controls. The use of PCR primers specific for *E coli* β-galactosidase provided a distinguishing marker for virus-derived DNA as opposed to endogenous β-galactosidase, verifying successful gene transfer. Furthermore, the absence of viral message other than the β-gal confirms that the vector is replication-defective and does not secrete viral products that affect islet function.

These DNA studies are consistent with transfer of the gene into islet cultures, but are not a guarantee of gene expression and protein production. The presence of virus derived β-galactosidase message in infected islets only, as well as detection of the protein product by specific antibody to *E coli* β-galactosidase and the functional ONPG assay of the protein, confirmed efficient gene expression. Furthermore, the large quantity of protein produced by infected islets is likely sufficient for biologic function, such as interference with the immune cascade.

The islet functional studies indicated that transferred gene expression, even in large amounts, did not compromise islet integrity. Islets exposed to vector have comparable insulin secretion in response to incubation in high-glucose media, and in response to theophylline. In addition, insulin staining patterns after transplantation of islets were unaffected by gene transfer. Furthermore, islets expressing recombinant protein functioned as well as control islets in reducing blood glucose levels after transplantation into diabetic mice. Thus, pretransplant genetic manipulation was well tolerated by the cultured cells and by transplanted islets.

Only short term in vitro and in vivo gene expression was documented in this study. After ten days of culture, the pattern of X-gal staining was not different from that at 24–72 hr, suggesting relative short-term stability of the transferred gene. In vivo, islets transplanted under the kidney capsule stained positively for β-gal after a month. The life span of the replication-defective adenovirus in islets is unknown, but it is likely that little of the Ad DNA is integrated in target cell genomes. In other systems, the stability of the transferred gene is variable, dependent upon cell permissivity (*26*), and probably on host proliferation rate. In nonreplicating cardiac muscle, for example, Ad vector expression has been reported to last for up to one year (*27*). Ongoing experiments are directed toward characterizing the life span of adenoviral vectors in islets in culture and after transplantation.

Although islet-directed gene transfer is successful using adenoviral vectors, the exact cellular target within the islet is not known. The cell subtype targeted by the vector may have implications for modifying cell surface antigenicity, and certainly for modifying cellular physiology. However, application of gene therapy to modulate regional immunity may not require the expression of the transferred gene by a specific cell type. For example, inhibition of the local immune response by increased concentration of inhibitor cytokines within islets is possible regardless of the cellular source of the protein. Nonetheless, current work is directed toward identification of the precise islet cellular target of the adenoviral vector.

In summary, this study established a model of successful and efficient gene transfer to cultured mouse islets. Use of adenoviral vectors to shuttle and induce expression of specific cDNAs in islets prior to transplantation provides a means to modulate the antiislet immune response.

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